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The effects of physiological levels of intermittent pressure on the development of articular cartilage in vitro

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The effects of physiological levels of intermittent pressure on the
development of articular cartilage *in vitro*

by

Scott Edward Carver

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Chemical Engineering

Major Professor: Carole A. Heath

Iowa State University

Ames, Iowa

1998

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CHAPTER 1. GENERAL INTRODUCTION

Introduction

The human body consists of a number of articulating joints, some of which, such as the knee and hip, frequently experience loads several times that of the body's own weight. When these joints fail, their movement often becomes limited and painful due to the breakdown of the articular cartilage within the joint. As this form of cartilage degrades, the tissue loses its ability to transfer and distribute the loads realized during normal daily activity. Articular cartilage degradation is the main cause of the most prevalent form of arthritis, osteoarthritis or OA, which affects more than 30 million people in the United States alone.

Since articular cartilage has a limited ability to repair itself [1], many therapies have been used to help alleviate the discomfort felt in arthritic joints. Recently researchers have described how ingestion of chondroitin sulfate and glucosamine, two components which make up articular cartilage, can help reduce the degradation of the tissue due to every day "wear-and-tear" [2]. If the breakdown of articular cartilage is detected in its early stages while the defect size is still small, the degradation can be arrested using localized cartilage cell injections [3], a therapy which was introduced commercially by Genzyme Tissue Repair. However, if the defect becomes too large, the damaged tissue needs to be surgically excised and a total joint replacement is often performed. The surfaces of these artificial joints are made up of low friction polymers having limited life-spans which often create more complications than they cure because of the need for additional surgeries. In order to completely restore the functionality of the joints affected by advanced arthritis, biological

grafts of articular cartilage created *in vitro*, with the same characteristics as tissue found *in vivo*, may be the best solution.

Over the last 40 years many researchers have helped to describe the composition and function of native articular cartilage so that the parameters for producing biochemically and mechanically functional graft tissue are known. In order to develop an adequate biological graft, the replacement tissue needs to be cultured in an environment which closely resembles a physiological system. Since articular cartilage experiences large pressure changes, has a continuous nutrient requirement, and must be maintained at a constant temperature, the development of a suitable culture environment must employ the principles of engineering. Perhaps the best engineering discipline equipped with the background to investigate this problem is chemical engineering.

Chemical engineers frequently study situations in which mass transfer, heat transfer, thermodynamics, and fluid flow need to be analyzed and optimized. With the appropriate biological background, a chemical engineer can design and create an *in vitro* reaction system having growth conditions similar to that of a native system. In recent years, a new area of chemical engineering has been developed, termed tissue engineering, which combines the biological sciences with engineering fundamentals to create functional repair tissue. Examples of tissues currently being investigated include skin, cartilage, nerve, liver, and kidney. Regenerated dermal tissue has already been developed commercially to be used for skin grafts and cosmetic therapies and has proven to be quite successful. Before articular cartilage can be developed commercially using the concepts of tissue engineering, the growth conditions which stimulate cartilage regeneration need to be completely determined.

In the last decade there has been much progress in optimizing the culture conditions needed to produce regenerated cartilage tissue with many of the same biochemical characteristics as native tissue. However, the culture conditions needed to produce mechanically functional repair tissue have yet to be determined. It was the goal of this study to develop a reaction system to regenerate articular cartilage which implements many of the same conditions found *in vivo* based on the hypothesis that these stresses would help create a fully functional repair tissue.

Dissertation Organization

This dissertation consists of three separate manuscripts which have been or will be submitted to the journals indicated below the title of each paper. These manuscripts are listed in this dissertation as chapter 2, chapter 3, and chapter 4. Preceding these manuscripts, in this chapter, are sections providing a general background on articular cartilage and an extensive literature review. Following the manuscripts, as chapter 5, are the general conclusions made from the results-to-date and recommendations for future research.

Background

Varieties of Arthritis

Arthritis has plagued the human race ever since mankind first walked the earth. Although there are several different types of arthritis, they all share some common symptoms. These include limited and/or painful movement of an afflicted joint along with some degree of inflammation (either primary or secondary). In some types of arthritis, damage to the underlying cartilage and/or bone can occur. The literal meaning of this often

debilitating disease comes from the Greeks who used it to mean inflammation of one or more joints. With the many different types of arthritis known today, there are four which occur most commonly in the world's population. These are ankylosing spondylitis, psoriatic arthritis, rheumatoid arthritis, and osteoarthritis [4].

Ankylosing spondylitis (AS) occurs more prevalently in men than in women, generally in individuals 20 to 30 years old [5]. This form of arthritis affects the spine and other joints of the trunk. In the latter stages of this disease the affected joints begin to fuse together, severely limiting their mobility. Discomfort may be noted in the thighs when walking or standing because minor changes in the sacroiliac joint within the pelvis may occur. Only in rare cases is this disease crippling; in fact most cases are so mild they will go undiagnosed for decades. Approximately 1 million Americans suffer from this form of arthritis [4,5].

Psoriatic arthritis (PA) involves a common skin condition known as psoriasis and the inflammation that occurs in its presence. Psoriasis causes the skin to assume an inflamed, reddish color. Also, the skin around the elbows and knees often becomes scaly in appearance. The inflammation of these joints caused by the psoriasis may cause some pain and discomfort but the overall effects are minor. In most cases, anti-inflammatory drugs and mild exercise are all that are needed for relief. Approximately 500,000 Americans suffer from this form of arthritis [4,5].

Rheumatoid arthritis (RA) is perhaps the most mysterious form of arthritis. Even though this form of arthritis has been clearly distinguished for nearly 200 years, the exact cause of the disease is still unknown. RA is thought to be an autoimmune response to an

unknown infectious agent. Typically the synovial membrane lining becomes inflamed as cells within the membrane divide and grow. As these inflammatory cells reach an elevated cellular density, the joint becomes swollen and tender to the touch. If the arthritic process continues for years, then enzymes secreted from the inflammatory cells can gradually digest the articular cartilage found within the joint, as well as the underlying bone. In its advanced stages RA can be a painful form of arthritis, but with early diagnosis and treatment most people tend to do well and lead normal lives. Approximately 7 million Americans, three-quarters of which are women, suffer from this form of arthritis [4-6].

Osteoarthritis (OA) is the most widely spread form of arthritis, affecting more than 30 million people in the United States [4-6]. When articular cartilage is damaged, either by a traumatic injury or gradual “wear and tear”, the tissue begins to degenerate irreversibly, causing the joint to fail. If diagnosed early enough, oral ingestion of proteins found in articular cartilage may slow or even stop OA’s progressive degeneration. Minor defects can also be treated with cartilage cell injections under a periosteal flap. These cells, called chondrocytes, excrete the proteins found in healthy cartilage and can help impede further degeneration of the damaged tissue. If the defect becomes too large, the only relief available is excision of the damaged tissue and its replacement with a low friction prosthesis. These artificial prostheses often have a limited life span and require major orthopedic surgery for their insertion and removal. Considering the large number of individuals affected by this disease, this is both a major medical and economic concern. Biological grafts made from full section regenerated cartilage tissue created *in vitro* have been proposed to help alleviate this growing problem. However, for the grafts to be able to withstand the repeated physiological

loads normally occurring in tissue found *in vivo*, these grafts must be developed in a manner so that their mechanical integrity will not be compromised over a long term period.

Varieties of Cartilage

Cartilage is a unique connective tissue combining resilience and rigidity with a high tensile strength and a great resistance to compressive and shear forces. This tissue is avascular, alymphatic, and has the lowest cell density of any tissue in the body. Found throughout the body, cartilage tissue serves different functions depending on its type and location. Minute features in its structure help distinguish among three separate cartilage types. These are elastic cartilage, fibrocartilage, and hyaline cartilage.

Elastic cartilage makes up the connective tissue found in the external ear, the auditory tube, epiglottis, and the corniculate and cuneiform cartilage's of the larynx [7,8]. Having an opaque yellowish color, this flexible form of cartilage is made up of an abundant amount of branching, elastic fibers composed of *elastin*. These fibers penetrate in all directions throughout the tissue and help provide this form of cartilage with its support structure characteristics.

Fibrocartilage is found in the intervertebral discs of the vertebral column, in the symphysis pubis, and in a few other joints [7,8]. It is made up of parallel collagenous bundles which encapsulate the cells in a thick, compact manner. This arrangement gives this form of cartilage the ability to be somewhat rigid and to handle compressive loads while still allowing some minor flexibility. It is thought that this cartilage type may be a transitional form between hyaline cartilage and dense connective tissue [7].

Hyaline cartilage is the most abundant form of cartilage found in the adult body. It makes up the cartilage of the costal, tracheal and laryngeal regions, nasal cartilage, and the articular cartilage found on the ends of bones [7,8]. Articular cartilage precedes bone formation and is the main focus of degenerative cartilage disease studies.

Articular Cartilage Morphology

Articular cartilage has a pearly blue color and is both shiny and translucent in its appearance. Covering the ends of articulating joints, it is a nearly frictionless tissue through which all but the most extreme compressive loads are distributed. Articular cartilage is made up of two separate phases: a solid phase made up of collagen fibrils, proteoglycans, and chondrocytes, and a fluid phase made up of interstitial water and electrolytes.

The collagen fibrils are the most abundant organic component of articular cartilage making up 50-80% of the dry weight and 15-22% of the wet weight [9]. The main type of collagen found in the tissue is type II, along with trace amounts of types VI, IX, and XI. The basic structural unit of all types of collagen is tropocollagen which is composed of three polypeptide chains. These α chains are coiled into left-handed helices which are further coiled about each other into right-handed triple helices. Each of these α chains are made up of amino acids sequenced in a known manner: glycine/proline/ Φ where Φ is either hydroxyproline or some other amino acid. Each tropocollagen molecule is rod-shaped having an approximate diameter of 1.4 nm and an approximate length of 300 nm [10]. These tropocollagen molecules polymerize into larger collagen fibrils in a unique manner. Each collagen fibril is formed from a quarter stagger array of tropocollagen giving the fiber its strength and its banded appearance when viewed using electron microscopy (Fig.1). Type II

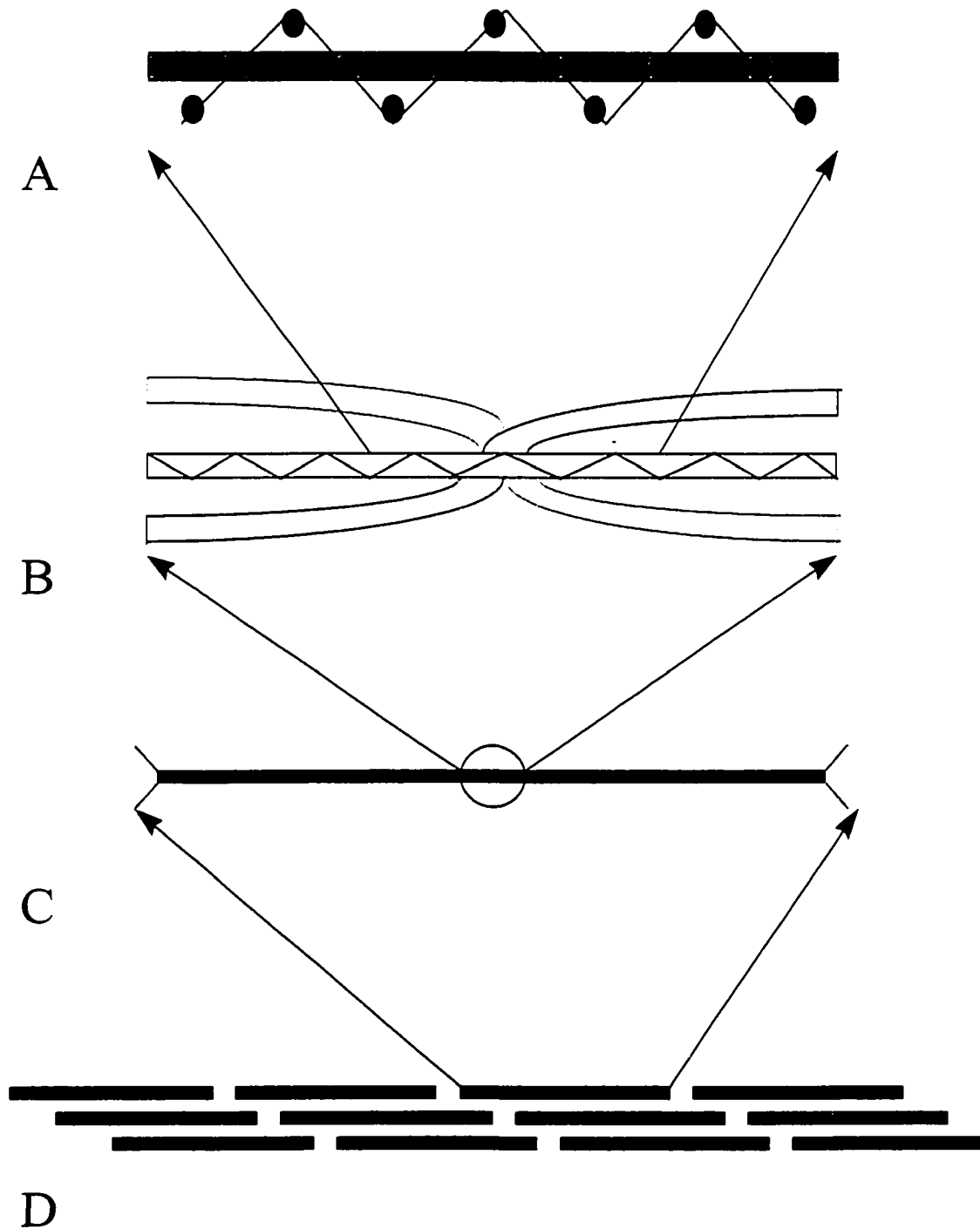
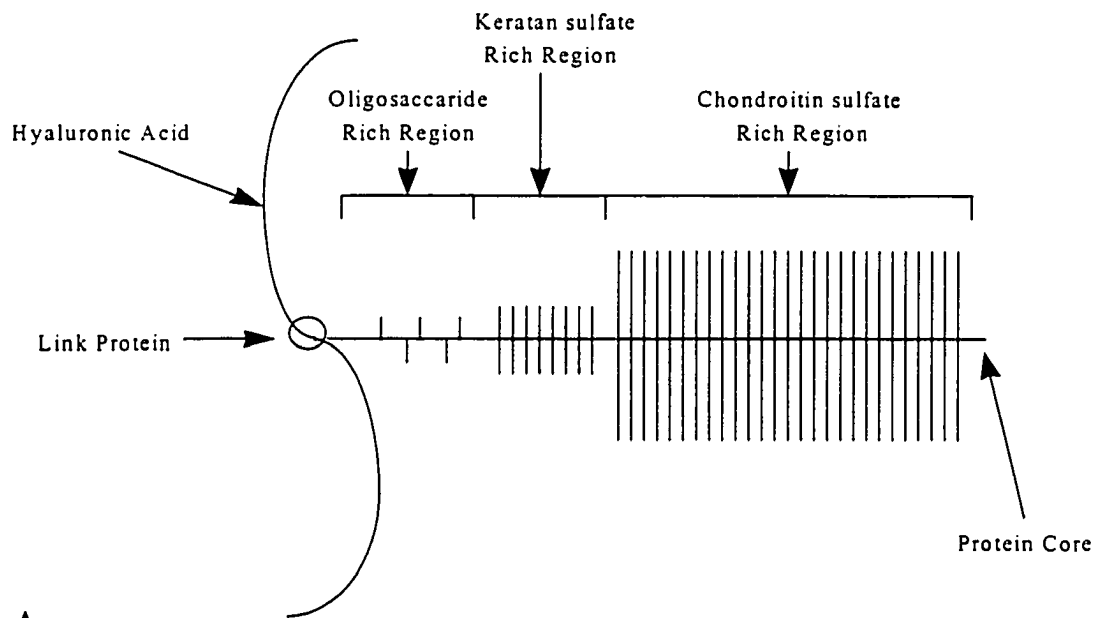


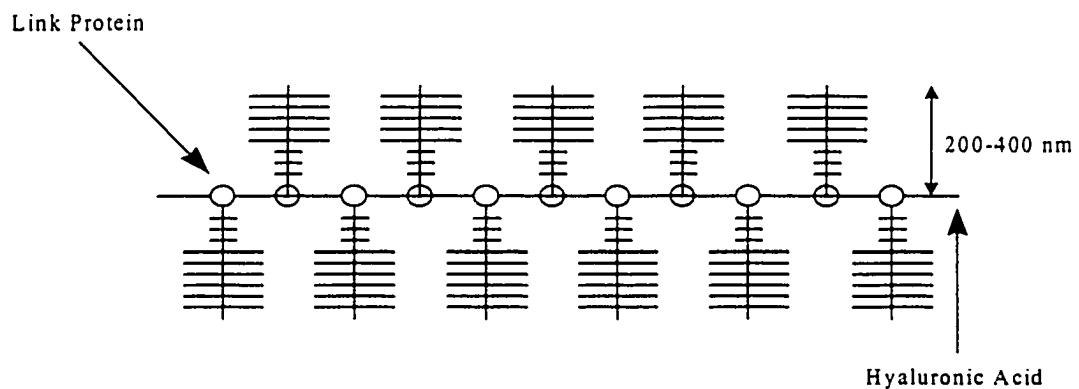
Figure 1. Hierarchical organization of a collagen fibril. A) The alpha chain. B) A triple helix. C) A tropocollagen molecule. D) The quarter stagger array of a collagen fibril. Adapted from reference 10.

collagen varies in diameter (20 to 200 nm) and there is no set length range. Biochemical evidence indicates that type II collagen covalently binds to type IX [11,12]. Since type IX is known to have a glycosaminoglycan chain attached to its $\alpha 2$ chain, this allows for a cohesive collagen-proteoglycan matrix to be established. The exact roles of types VI and XI collagen in the extracellular matrix are still unknown as of now.

Proteoglycans make up 4-7% of the wet weight of articular cartilage [9]. While interacting with the collagen fibrils to make up the extracellular matrix, they also help with water retention throughout the tissue. The structure of a proteoglycan molecule consists of a core protein to which oligosaccharides and two types of glycosaminoglycans are attached (Fig. 2a). When an animal is in its beginning years of development nearly all of the glycosaminoglycan present is in the form of chondroitin sulfate. However, as an animal matures, nearly one-third of the glycosaminoglycan develops into keratan sulfate with the other two-thirds consisting of chondroitin sulfate. The typical length of a proteoglycan molecule can range from 200-400 nm. At the N-terminal region of the core protein, the proteoglycan attaches to a hyaluronate chain via a link protein. When enough proteoglycans attach to an hyaluronic acid chain a proteoglycan aggregate is formed (Fig. 2b). The majority of the proteoglycans present in articular cartilage are the large aggregating type (50-85%) while the remainder of the proteoglycans are the large non-aggregating type (10-40%) and smaller distinct forms [9]. Chondrocytes are the only cell type found in articular cartilage. Because cartilage has no blood or lymph supply, the chondrocytes get all their nutrients from



A



B

Figure 2. A) The organization of a proteoglycan monomer, and B) a proteoglycan aggregate composed of many monomers attached to a hyaluronate chain. Adapted from reference 9.

the synovial fluid surrounding the articular joint. Found within cavities called lacunae, these cells are known to produce and excrete type II collagen and proteoglycans into the surrounding extracellular matrix. When chondrocytes are grown in a monolayer they dedifferentiate into fibroblastic like cells, losing their three-dimensional characteristics. As fibroblasts, these cells produce type I collagen that is typically found in fibrocartilage. When removed from a monolayer the fibroblasts differentiate back into chondrocytes and will remain that way as long as they are cultured in a three-dimensional environment.

Interstitial water, along with its dissolved electrolytes, make up 60-85% of the wet weight of articular cartilage. When cartilage is compressed the water within the tissue is pushed out through interstitial pores into the surrounding synovial fluid. When the compressive force is released, the tissue acts as a sponge, pulling the water back in along with nutrients necessary for cell metabolism. The majority (~65%) of the water present in articular cartilage associates with the negatively charged groups on the proteoglycans, with the balance of the water found in the intrafibrillar and intracellular compartments of the collagen and chondrocytes, respectively.

Articular Cartilage Structure

Articular cartilage is organized into a unique trizonal arrangement thought to give the tissue its ability to withstand large compressive loads (Fig. 3). The top layer, called the *superficial tangential zone*, comprises 10-20% of the tissue. In this layer, the collagen fibrils

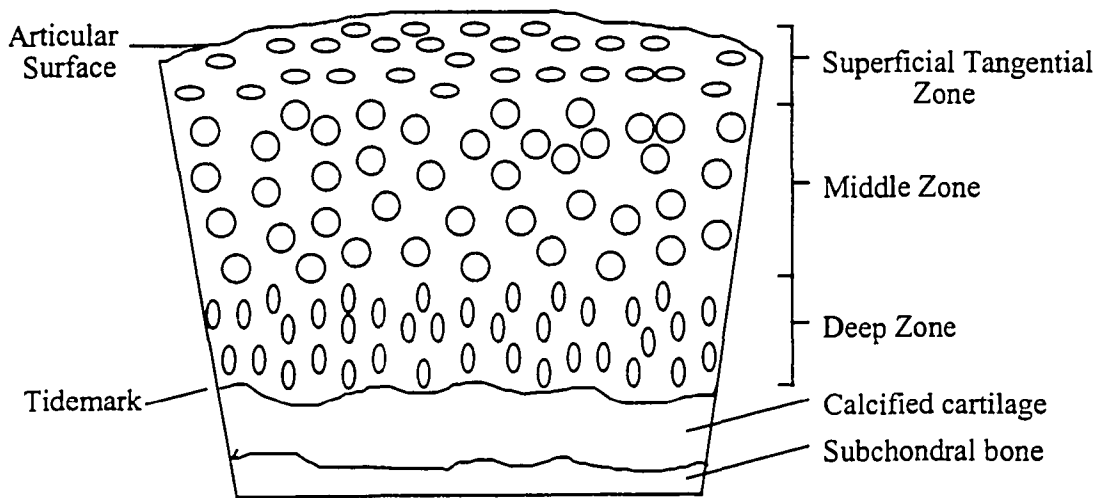
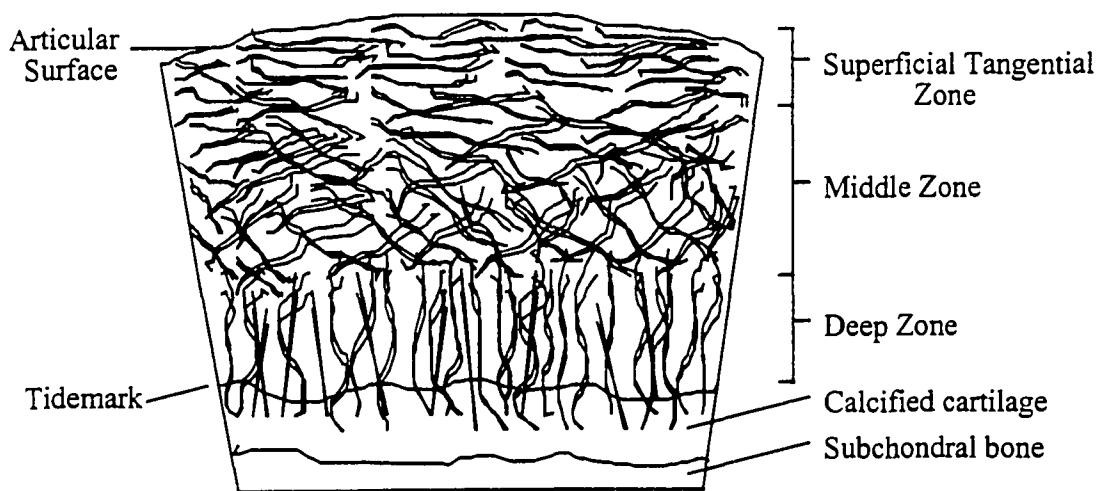
**A****B**

Figure 3. The organization of A) cells and B) collagen throughout the three zones of articular cartilage.

are orientated parallel to the articular surface in order to withstand shear forces that the tissue endures during normal joint movements. The chondrocytes, dispersed within the collagenous matrix, appear flattened in this region. The *middle zone*, comprising 40-60% of the tissue, is composed of collagen fibrils orientated at various angles. The fibrils at the top of the middle zone have a very shallow angle with respect to the articular surface. Proceeding deeper through the middle zone, the fibril angle continuously increases. Since the average collagen fibril angle in the middle zone is 45° , the chondrocytes found in this zone appear more rounded. This region is a transitional zone between the *superficial tangential zone* and the *deep zone*. The *deep zone* comprises 30% of articular cartilage tissue. The collagen fibrils in this region, orientated perpendicular to the articular surface, serve two purposes. First, this arrangement helps absorb compressive loads applied to the tissue, and second, it helps anchor the tissue to the underlying subchondral bone. With these three unique layers, articular cartilage can handle a wide variety of compressive and shear forces.

Literature Review

Characterization of Articular Cartilage

Even though osteoarthritis was clearly distinguished as a separate disease from rheumatoid arthritis during the beginning of the twentieth century [4], articular cartilage was not studied for decades afterwards. All that was known, until the second half of the twentieth century, was that articular cartilage has a very limited ability to repair itself [1]. From 1952 to 1979, the main focus of articular cartilage research was characterizing the makeup, function and behavior of the tissue. Articular cartilage was first characterized

histochemically by Eichelberger et al. [13-15] in a three-part study ranging from 1952 to 1959. In 1954, Belanger [16] showed that radioactive sodium sulfate became incorporated first into chondrocytes, and then into the extracellular matrix. Revel and Hay [17] confirmed that the cells were the precursors to the extracellular matrix while investigating collagen synthesis. These two studies were the first to show proteoglycan and collagen synthesis and secretion from the chondrocytes, respectively.

Since very little was known about the morphology of articular cartilage in the early 1960's, researchers began to study the individual components of the tissue, as well as the tissue's behavior. Many studies focused on the *in vitro* culture of chondrocytes. In 1966, Coon [18] successfully cultured chick chondrocytes and first described the change in phenotype typical of the cells when grown in a two-dimensional environment (a monolayer). Manning et al. [19] were able to isolate and culture human chondrocytes in the following year. Chondrocytes were first cultured in a three-dimensional environment by Horwitz and Dorfman in 1970 [20]. This study showed that chondrocytes would not dedifferentiate when grown in a support matrix. While others described culturing chondrocytes *in vitro* in the following years [21-23], as well as chondrocyte characteristics [24], it was not until 1978 that Benya et al. [25] made an important discovery. They showed that chondrocytes grown in a monolayer produce type I but not type II collagen, which is found in fibrocartilage and not in articular cartilage. This outlined the importance of culturing chondrocytes in three dimensions in order to develop healthy articular cartilage.

The characterization of articular cartilage tissue, both healthy and osteoarthritic, was thoroughly researched between 1965 and 1979. Bollett and Nance [26] first described the

biochemical characteristics of normal and osteoarthritic articular cartilage. They showed that osteoarthritic tissues typically exhibit an increase in water content and a decrease in proteoglycan content. To set a standard for grading osteoarthritic tissue, Mankin et al. [27] introduced a system to classify the degree of change observed at the micro-level. The system focused on cellularity, cell number, the appearance of cartilage fibrillation, and proteoglycan loss. In 1967, Edwards [28] described some of the physical characteristics of articular cartilage. Edwards described the behavior of interstitial water movement throughout the tissue when subjected to a compressive load. At this time the composition of interstitial water was defined [29]; however, little was known of the fine structure of articular cartilage through which it flowed. Bullough and Goodfellow [30] first described the minute appearance of articular cartilage which was followed by microscopic studies that helped further define the tissue's structure [31,32].

In the mid to late 1970's researchers began to study the effects of mechanical stress and pressure on articular cartilage. Mansour and Mow [33] first subjected the tissue to high pressures while studying its permeability. Caterson and Lowther [34] first reported that proteoglycan synthesis increases when articular cartilage is subjected to a mechanical stress. It was not until the following year that Veldhuijzen et al. [35] subjected chick chondrocytes *in vitro* to intermittent compressive forces. They showed that chondrocytes deposit more of their matrix when subjected to intermittent pressures and that the nuclear and cell dimensions increased. Even though the chondrocytes were only cultured for short time periods (24 to 48 hours), this study showed the importance of using intermittent compressive forces to maximize matrix production.

Enhancing Cartilage Matrix Production

By 1979, articular cartilage and its components had been well described, however, studies on enhancing tissue production and repair had only begun. Studies to determine the roles of type II collagen and proteoglycans continued to be performed using X-ray diffraction [36] and electron microscopy [37,38] to further define the tri-zonal arrangement of the collagen fibrils. Hardingham [39] first described the structure of proteoglycans and how they are organized throughout the extracellular matrix. Muir suggested that the proteoglycans organize the intracellular matrix of articular cartilage [40] and went on to describe how proteoglycan aggregates are formed by chondrocytes [41]. Poole [42] described how the loss of proteoglycans in the extracellular matrix contributes to osteoarthritis. He found that proteoglycans play a vital role in fluid retention within the extracellular matrix, and that without them articular cartilage cannot remain intact. As the importance of proteoglycans was realized, many researchers explored the growth conditions needed to enhance their production. Also, the growth conditions needed for type II collagen production by the chondrocytes was under investigation. These two interests were explored separately.

In a follow-up study, Benya and Shaffer [43] cultured chondrocytes in agarose gels in order to assess environmental effects on collagen production. They showed that chondrocytes grown in a monolayer will re-express the differentiated collagen phenotype when grown in a three-dimensional environment. Kimura et al. [44] confirmed this finding while culturing chondrocytes in collagen gels. These studies further defined the importance of culturing chondrocytes in a three-dimensional environment to generate the structural components necessary for articular cartilage regeneration.

Although gels and agars were ideal for *in vitro* culture of chondrocytes, a support matrix that was biosorbable was necessary if regenerated cartilage was ever to be implanted into an *in vivo* environment. Vacanti et al. [45] studied the effect of three synthetic polymers on cell growth and viability. Polyorthoesters, polyanhydride and polyglactin were all cultured with hepatocytes, pancreatic islets and intestinal cells. Vacanti et al. found that the cell viability was greatest on polyglactin (over 90%), followed by polyorthoesters (around 80%) and polyanhydride (less than 10%). Perhaps it was these promising results with polyglactin, coupled with a study comparing polyglactin and polyglycolic acid done by Craig et al. [46], that later led Cima et al. [47] to first use fiber-based felts of polyglycolic acid as a support structure. Because of its biosorbable properties, polyglycolic acid has become the most used support structure for regenerating articular cartilage to date (more discussion later).

Many researchers explored the influence of mechanical loading on cartilage cell culture in the hopes of enhancing proteoglycan synthesis. The mechanical properties of articular cartilage were well described within this decade. Paul [48] was one of the first to report on how cartilage can be subjected to forces several times that of the body's own weight during dynamic loading within an articulating joint. Armstrong and Mow [49] described how the material properties of articular cartilage can change as the tissue degenerates. Two years later they described how healthy articular cartilage acts as a solid swollen with water and has the ability to withstand a wide range of stresses and strains [50]. Although the mechanical characteristics of articular cartilage were becoming well known, the influence of mechanical force on cartilage matrix formation was not fully understood.

Jones et al. [51] first described how continuous mechanical force could actually suppress the ability of a chondrocyte to synthesize proteoglycans. Copray et al. [52] later confirmed that compressive forces can be detrimental to articular cartilage formation unless applied in a certain manner. When chondrocytes are subjected to *intermittent* compressive forces they increase their synthesis and content of proteoglycans, which was confirmed in a follow-up study by Veldhuijzen et al. [53]. When the level of intermittent compressive force enters the *in vivo* domain, the rate of proteoglycan synthesis increases until it exceeds physiological levels [54]. However, for articular cartilage to be subjected to *in vivo* pressures, compression must be done in a hydrostatic or physical manner to avoid compressing the gas phase. Lippiello et al. [55] first subjected bovine and human articular cartilage to hydrostatic pressures up to the physiological domain. They found that proteoglycan synthesis decreased by 50% as the hydrostatic pressure level was increased from 0.5 MPa to 2.0 MPa. However, as the pressure level was further increased to the physiological domain (2.5 MPa), the rate of proteoglycan synthesis increased by 55%. This study emphasized the importance of using physiological pressures to further enhance matrix production. As the decade came to an end, chondrocyte response to mechanical stimuli continued to be investigated [56,57]; however, combining the conditions enhancing cartilage regeneration was yet to be done.

Regeneration of Articular Cartilage

During the early 1990's many studies involving articular cartilage were focused on using explant cultures exposed to mechanical stimuli. Hall et al. [58] confirmed proteoglycan synthesis was enhanced when subjected to hydrostatic pressures in the

physiological domain. By studying a hydrostatic pressure range of 2.5 to 50 MPa, they concluded that matrix production was stimulated from 3.0 to ~18.0 MPa; however, as the pressure level exceeded 18.0 MPa, the rate at which proteoglycans were synthesized steadily decreased. The effects of applying hydrostatic pressure, both positive and negative, under cyclic compressive loads were first described by Parkkinen et al. [59] and Suh and Woo [60], respectively. Both studies showed that cyclic hydrostatic pressures significantly influenced the production of extracellular matrix within their respective explant cultures. However, others showed that compression applied in a physical manner inhibited the rate of proteoglycan synthesis. Sah et al. [61] examined the effects of applying physical compression to cartilage explants and found that newly formed proteoglycans were not retained within the extracellular matrix. Burton-Wurster et al. [62] later subjected canine explants to both static and intermittent physically applied compressive loads ranging from 0.5 to 1.2 MPa for up to 18 hours. They found that matrix production was inhibited in static cultures and did not differ from intermittent cultures. These five studies showed that hydrostatic pressurization, as opposed to physical compression, was a better means of producing the extracellular matrix of articular cartilage.

As studies involving cartilage explants continued, Cima et al. [47] introduced an attractive and potentially better way to culture articular cartilage. They isolated liver and cartilage cells from healthy tissue and transplanted these cells onto biosorbable polymer support matrices. Two polymers were investigated in their study: a polyglycolic acid (PGA) fiber-based felt and a copolymer of polylactic acid and polyglycolic acid (PLGA) cut into braided threads. After being grown subcutaneously in nude mice, the *regenerated* articular

cartilage was excised and analyzed. Histological sections showed evidence of cartilage formation very similar in appearance to normal human fetal cartilage. Because of their biocompatibility, degradability and processability characteristics, these polymer support matrices offer many advantages. PGA and PLGA have both been approved by the FDA and have been used as suture materials for years. By varying the ratios of one polymer to the other in a support matrix, controlling the rate at which the polymer degrades is possible. These polymers degrade by hydrolysis, forming natural intermediates, which are removed from an *in vivo* system through normal metabolic pathways. Perhaps the most attractive quality of using these support matrices is that they can be processed into a variety of shapes and sizes using melt and solvent techniques. Combining these features makes it possible to develop full section biological grafts to use as replacement tissue for those suffering from osteoarthritis.

The possibility of creating predetermined shapes and sizes of regenerated articular cartilage led many researchers to explore this option. Klompmaker et al. [63] attempted to use an aromatic porous polyurethane as a support matrix for regenerating rabbit and dog articular cartilage. Because this polymer can release toxic degradation products, they also used a copolymer of L-lactide and ϵ -caprolactone with dog chondrocytes. They found that using these polymers *in vivo* resulted in the formation of fibrocartilaginous repair tissue which seemed to function adequately in the dog but showed signs of degeneration in the rabbit. This study further confirmed the importance of using biocompatible polymers when culturing articular chondrocytes.

At the Massachusetts Institute of Technology (MIT), researchers focused on using nonwoven polymer meshes of polyglycolic acid (PGA) and porous poly(L)lactic acid (PLLA) for neocartilage formation. Freed et al. [64] used these polymers to culture articular bovine and costal human cartilage *in vitro* and *in vivo*. They found that the cell growth rate was twice as high on the PGA as it was on the PLLA substrates. The difference in growth rates was attributed to the polymers' geometry and degradation rates. Freed et al. [65] later seeded human costal chondrocytes on PGA and cultured them in petri dishes and spinner flasks. Scaffolds in the well-mixed spinner flasks did not suffer from the mass transfer limitations that are commonly associated with static culture conditions. Freed et al. [66] went on to describe the growth kinetics of chondrocytes cultured on varying thicknesses of PGA. As the scaffold thickness increased, the cell growth rate decreased in static cultures. However, in well-mixed cultures, high cell growth rates were maintained over seven weeks. These two studies outlined the importance of culturing the cell/polymer constructs in an environment which minimizes the diffusional limitations of nutrients to the chondrocytes. Freed et al. [67] also demonstrated the orientation of the tri-zonal arrangement in cell/polymer constructs in a study focusing on scaffold composition. Scaffolds from mixed cultures were found to have a superficial tangential zone with collagen orientated flat to the surface and a deep zone with collagen orientated perpendicular to the surface. They attributed the formation of the layered arrangement to the mixed cultured environment in which the tissue was regenerated. Kim et al. [68] later cultured bovine articular chondrocytes on PGA scaffolds which were configured into four unique shapes: a triangle, a rectangle, a cross, and a cylinder. They concluded that chondrocytes cultured onto their respective

scaffolds will synthesize the extracellular matrix within the confines of the predetermined shape. This study confirmed that it was possible to develop articular cartilage into given shapes and sizes.

Although these researchers at MIT were successfully culturing chondrocytes on PGA to regenerate articular cartilage, the tissue they were developing probably was not fully functional within an *in vivo* system. This perhaps was a result of culturing chondrocytes in an environment where they were not subjected to the compressive forces that they normally sense *in vivo*. In 1994, Magari [69] and Heath and Magari [70] concluded a study that was the first to combine culturing cell/polymer constructs with the application of intermittent pressurization. She found that by applying a 50 psi intermittent pressure to bovine chondrocytes seeded onto PGA, the constructs developed appeared qualitatively stronger compared to control samples. Fluorescence microscopy qualitatively confirmed that pressurized samples consisted of more type II collagen than control samples. However, results involving the production of the extracellular matrix in pressurized samples varied, motivating future studies to be conducted at greater intermittent pressure levels.

Current Advances and New Techniques

During the last few years there has been a tremendous increase in interest in the repair and replacement of articular cartilage. At MIT, Freed et al. have continued to examine the effects of mixing [71-73], the kinetics of mass transfer [74], *in vitro* culture [75] and *in vivo* implantation [76]. Their results confirm that articular cartilage constructs can be developed in an *in vitro* environment when nutrient requirements for the chondrocytes are fulfilled. In a recent publication regarding mixing [73], they introduced a new approach for seeding PGA

scaffolds with articular chondrocytes. “Dynamic cell seeding” involves immobilizing PGA scaffolds within a spinner flask and placing the chondrocytes in the stirred medium. Since the cells have a high affinity for binding to the PGA, they are distributed throughout the suspended scaffolds. This technique has proved to be especially useful as the thickness of the scaffold is increased since mixing ensures that cells penetrate the scaffold rather than adsorbing solely to the polymer surface. In their most current work, Freed et al. describe how dynamic mixing at high cell concentrations (1.33×10^5 to 6.67×10^5 cells/cm³) promoted the formation of cell aggregates ranging from 20 to 32 μm in diameter [77]. These aggregates were found to enhance the rate of cell attachment to the PGA scaffolds so that all cells adhered to the polymer within one day. Other researchers at MIT have explored the possibility of using regenerated cartilage for reconstructive and aesthetic surgery of the nose [78]. By creating anatomical models of nasal septal cartilage, they developed polymer constructs showing sufficient structural stability *in vitro* and *in vivo*, opening up new possibilities for facial plastic surgery.

Studies focusing on chondrocyte response to variations in the culture conditions are still being conducted. Ishizaki et al. [79] demonstrated how chondrocytes produce autocrine signals when cultured at high cell densities, enabling them to survive in the absence of serum or other exogenous proteins. However, at low cell densities they found that the chondrocytes die by apoptosis. Only by using medium from high cell density cultures, serum or a combination of known growth factors, can chondrocytes survive at low cell densities.

Others have demonstrated how chondrocytes respond to decreases in culture temperature while being stored. Kim et al. [80] described the effects of culturing

chondrocytes at 4°C. By using an appropriate medium they were able to store chondrocytes for up to 4 weeks, at which point approximately 47% of the cells were still viable. These cells also maintained their ability to form new cartilage when cultured in polymer matrices. Ohlendorf et al. [81] determined how well chondrocytes in articular cartilage explants survive during cryopreservation. Chondrocyte survival was found to be limited to the superficial layer of the explants, with or without the addition of dimethyl sulfoxide. Tavakol et al. [82] described similar findings while characterizing freeze-thawed articular cartilage at the ultrastructural level.

Other ultrastructural assessments of chondrocytes have been made in cultures using new polymer systems. Reissis et al. [83] demonstrated the use of poly-ethyl-methacrylate (PEMA) and tetra-hydrofurfuryl-methacrylate (THFMA) as a potential biomaterial for regenerating articular cartilage. As with previous studies with PGA, they found that these materials were suitable for cartilage repair, creating more possibilities for the development of regenerated tissue. Frondoza et al. [84] recently described culturing chondrocytes in microcarrier suspension cultures. Chondrocytes were found to attach to the microcarriers, helping them to retain their rounded shape and producing the appropriate extracellular matrix.

At the University of Texas Health Science Center, Athanasiou et al. [85] continued characterizing PLA-PGA orthopedic implants. They reported on the degradation times of seven different combinations of PLA and PGA. Ranging from seven days to four years, these degradation times allowed researchers to customize orthopedic implants by choosing a polymer combination best suited to the situation. In a follow-up study, Thompson et al. [86]

subjected polymer constructs, composed of 50%-50% PLA-PGA seeded with chondrocytes, to dynamic compressive loads. Using a physical compression apparatus over a 42 day period, they found that dynamic loading enhances the degradation rate of the copolymer.

At Advanced Tissue Sciences in La Jolla, California, researchers explored several different aspects of tissue regeneration using rabbit and bovine chondrocytes. Dunkelman et al. [87] constructed a closed bioreactor system in which PGA scaffolds seeded with rabbit chondrocytes were cultured. They found that their regenerated tissue had similar biochemical characteristics to constructs described in previous studies. The advantage of the biosystem they developed was that the scaffolds could be cultured for several weeks without interruption. Because the constructs could be cultured for long-term durations, the regenerated tissue was allowed to develop more fully. Zimmer et al. [88] explored the effects of using the TGF- β growth factor. TGF- β was found to increase the proliferation rate of chondrocytes in a monolayer as well as to increase the ability of the chondrocytes to produce their extracellular matrix when cultured on PGA. In a recent study, Grande et al. [89] described culturing chondrocytes on four different support materials. These materials were composed of type I collagen, nylon, PGA and Vicryl. PGA cultures showed the greatest production of sulfated glycosaminoglycans while type I collagen cultures produced the greatest amount of type II collagen. These constructs were also cultured in a closed-loop system which improved the rates of matrix production.

Over the past couple decades researchers have used chondrocytes from animals such as cows, rabbits and dogs because they are easily attainable. Although much has been learned about chondrocyte behavior, tissue regenerated from these cells has little practical

use. Because human articular cartilage can be difficult to obtain, some researchers have started investigating the regenerative properties of equine cartilage. Horses often suffer from joint disease and replacing damaged tissue with functional regenerated tissue would at times be more attractive than euthanasia. Equine cartilage has recently been shown to have similar biochemical and mechanical characteristics as human articular cartilage [90]. At veterinary colleges across the country, researchers have made much progress with understanding joint disease in the horse. At Colorado State University, Wayne McIlwraith has been a leading force in diagnosing joint defects in the horse. In his recent publication, McIlwraith [91] thoroughly describes everything from equine joint anatomy to current ongoing research. Researchers such as Rory Todhunter, George Lust and Alan Nixon have been examining full-thickness articular cartilage defects at Cornell University for the past several years. In 1994, they reported on a study involving resurfacing articular cartilage defects with a chondrocyte-fibrin matrix [92]. This resurfacing technique helped improve the damaged cartilage surface compared to the control samples and generated a significantly greater proportion of type II collagen. One other publication by this group involved the effects of exercise on the repair of articular cartilage defects [93]. They documented that postoperative exercise was beneficial to the development of cartilaginous repair tissue in large defects in equine joints. Palmer et al. [94], at Ohio State University, reported on the biomechanical properties of articular cartilage in exercised and nonexercised horses. They found that at given sites (joint surfaces) exercise increased the permeability constant and Poisson's ratio, but did not affect the aggregate modulus. They recommended that further studies be conducted involving exercise to better characterize its effects.

New therapies have been introduced recently that have been shown to impede degradation of articular cartilage. In “The Arthritis Cure”, Theodosakis et al. [2] described how orally ingesting glucosamine and chondroitin sulfates helps the body to repair damaged or eroded tissue. They claimed that by supplying the body with these proteoglycan components, damaged tissue will not continue to lose these water-retaining components as they typically do during normal degeneration. At Genzyme Tissue Repair, researchers have developed a new product called Carticel. By using a method developed by Brittberg et al. [3], Genzyme created an injectable matrix-rich solution which is placed under a periosteal flap to regenerate small defects in articular cartilage. These techniques have been shown to be quite effective for arresting early cartilage degradation, but may prove to be ineffective for the repair of larger defects.

Over the past few years, researchers have experimented with new types of equipment to quantitatively and qualitatively assess articular cartilage. Guilak and Mow [95] used confocal microscopy to detect three-dimensional changes in cell shape and volume while the matrix was loaded by compression. Using a finite element model they were able to describe the mechanical effects on cellular elastic properties, cell shape, intercellular spacing and the presence of the pericellular matrix *in vitro*. Many nondestructive forms of analysis *in vivo* have also been described. Steele et al. [96] used magnetic resonance imaging (MRI) to develop a three-dimensional representation of the tibiofemoral (knee) joint. In the following years Marshall et al. [97] and Eckstein et al. [98,99] both used MRI to quantify articular cartilage thickness. These studies accurately and reproducibly measured articular cartilage volume and they provided the means to noninvasively assess changes in volume normally

resulting in the early stages of osteoarthritis. Cohen et al. [100] further described using MRI to obtain 3-D surface topographies, thicknesses, and contact areas of articular cartilage which they validated using stereophotogrammetry. At the University of California, Santa Barbara, Petersen et al. [101] have developed a hollow fiber chondrocyte bioreactor which is compatible with nuclear magnetic resonance (NMR) to allow noninvasive study of neocartilage formation. Initial results with this system indicate that, after a one month growth period, the NMR properties of the cultured neocartilage were found to correlate well with known histological data. Chiang et al. [102] recently described the use of a ultrasonic technique to better detect the surface fibrillations which are characteristic of osteoarthritic cartilage. After validating this new technique with laser-based confocal microscopic imaging, Chiang et al. claimed that ultrasonic assessment allows better resolution than current MRI methods. Another technique currently being investigated to reconstruct three-dimensional representation of articular cartilage is ultrasound microscopy (UM). Harasiewicz et al. [103] and Saied et al. [104] both described using this technique as a means for early detection of osteoarthritic changes within articular cartilage. UM allows the user to obtain information about the articular cartilage surface, subsurface structures and overall thickness. Other microscopic techniques have also been developed in recent years to better characterize articular cartilage. Jurvelin et al. [105] have developed an optical method which allows a direct determination of Poisson's ratio of tissue at equilibrium. Also, the aggregate modulus, H_a , and Young's modulus were determined using the equilibrium behavior of cartilage disks in unconfined and confined ramp-stress relaxation tests. Another technique recently developed to determine cartilage surface profiles and tissue thickness is multi-station

digital photogrammetry (DPG) and thin-plate spline surface interpolation (TPS). Ronsky et al. [106] and Boyd et al. [107] have used these techniques to better visualize joint anatomy and develop an excellent mathematical model of the joint surface. At MIT, Berkenblit et al. [108] have developed an electrokinetic surface probe which applies currents to the surface of articular cartilage and measures the resulting stress. This technique gives yet another option for detecting degradative changes in articular cartilage by non-destructively assessing its material properties. These new techniques should allow researchers to further characterize articular cartilage and increase our understanding of how this tissue functions and reacts to changes in its environment.

In order to better understand how mechanical forces normally influence development and maintenance of articular cartilage *in vivo*, researchers have continued to investigate systems implementing many different loading conditions. In a recent study, Lee and Bader [109] applied physiological loads to chondrocytes seeded in agarose. They found that static strains inhibited the synthesis of glycosaminoglycans while dynamic strains stimulated their synthesis, confirming that chondrocytes regenerate their extracellular matrix when loaded in a manner similar to that found *in vivo*. Shepherd and Seedhom [110] applied physiological loading rates to articular cartilage in order to better characterize the tissue mechanically. Using human knee joints, they found that the compressive modulus of articular cartilage was much greater than previously reported. At 20 milliseconds, the modulus was found to be between 4.4-27 MPa, a value 32 to 75% greater than its value obtained at 2 seconds. Using unconfined compressive loading, Suh and DiSilvestro [111] were able to better describe the biphasic poroviscoelastic behavior of articular cartilage. They noted a considerable

discrepancy in the stress relaxation response between the biphasic model prediction and the actual tissue behavior, allowing them to better describe the physical characteristics of cartilage behavior after pressure release. Suh et al. [112] went on to determine the effects of negative intermittent pressures on chondrocyte biosynthesis. They found that negative pressure increased the synthesis of proteoglycan and non-collagenous protein synthesis by 40 and 17%, respectively, demonstrating that chondrocyte biosynthesis has a similar response to positive and negative loading.

Physical deformation studies involving cartilage explants have helped characterize the metabolic response of chondrocytes to compressive loading. Torzilli et al. [113] have developed a mechanical explant system which is capable of loading cartilage from 0.5 to 24 MPa. Using this system, they found that statically and dynamically applied compressive stress decreased proteoglycan biosynthesis at all loading time intervals. Their results indicated that the cyclically loaded explants may have been dominated by the static component of the dynamic load. Wong et al. [114] used static compression to investigate cellular responses at different depth-zones, from the articular surface to the cartilage/bone border. Cell biosynthesis was found to vary at different levels within the tissue depending on the axial strain created by the applied strain. Buschmann et al. described the effects of compressive loads on biosynthetic activity of chondrocytes cultured in agarose disks [115] and aggrecan synthesis in explant cultures [116]. These two studies showed that dynamic compression is most effective when chondrocytes retain their three-dimensional characteristics and that compression may stimulate an intracellular signal transduction pathway to which chondrocytes respond. In recent years, researchers have been trying to

better understand how chondrocytes sense pressure. One hypothesis is that mechanoreceptors stimulate the stress activated protein kinase pathway which causes an increase in matrix production. Wright et al. [117] described how $\alpha 5 \beta 1$ integrin plays an important role as a chondrocyte mechanoreceptor. Using pharmacological inhibitors, they have demonstrated that both tyrosine protein kinase and protein kinase C activities are important in the transduction of the electrophysiological response to mechanical stimuli. This study was the first step in explaining how chondrocytes sense pressure and, as the years progress, chondrocyte signaling characteristics will undoubtedly continue to be revealed.

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CHAPTER 2. A SEMI-CONTINUOUS PERFUSION SYSTEM FOR DELIVERING INTERMITTENT PHYSIOLOGICAL PRESSURE TO REGENERATING CARTILAGE

A paper accepted by Tissue Engineering

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Abstract

A semi-continuous compression/perfusion system has been custom made to allow the application of intermittent hydrostatic pressure, at physiological levels, to regenerating tissues over the long term. To test the system, isolated foal chondrocytes were seeded in resorbable polyglycolic acid meshes and cultured in the system for five weeks. The cell/polymer constructs were subjected to an intermittent hydrostatic pressure of 500 psi and were fed semi-continuously. Assays of the resulting tissue constructs indicate that the reactor supports cartilage development and that physiological intermittent compression enhances the production of extracellular matrix by the chondrocytes. The concentrations of sulfated glycosaminoglycan were found to be at least twice as high as those in control (unpressurized) samples. A correlation between the sulfated glycosaminoglycan content and the compressive modulus in pressurized, but not control, samples suggests that physiological intermittent pressurization not only enhances the production of extracellular matrix but may also influence matrix organization resulting in a stronger construct.

Introduction

Articular cartilage, which covers the ends of all synovial joints, is composed of chondrocytes, proteoglycans, collagen (primarily type II), and water. Sulfated glycosaminoglycans (GAG), localized within the collagen network, keep the tissue hydrated and resistant to compression, while cross-linked collagen fibrils provide resistance to shear and tension. The large water content gives cartilage the ability to distribute loads throughout the highly porous matrix and over the joint surface. During normal daily function, articular cartilage can be repeatedly subjected to forces up to several times body weight.

Articular cartilage, despite its tremendously important function of providing articulating joints with a nearly frictionless, weight-distributing surface for the transference of forces between bones and joints, has a very limited ability to repair itself with biomechanically functional tissue.^{1,2} One particularly promising solution is to develop functional replacement cartilage *in vitro* that can be implanted *in vivo*. Although progress has been made in establishing tissue which is biochemically similar to native cartilage, the specific conditions of mechanical force believed to be responsible for development of the native material structure have not yet been determined. Knowledge of these conditions is crucial to the development of functional replacement tissue (i.e., with the ability to bear and distribute weight).

The phenotypic nature of the chondrocyte is a direct result of its growth environment. Without a three-dimensional support structure *in vitro*, the chondrocyte will dedifferentiate into a fibroblast-like cell and begin producing fibrous, rather than articular or weight-bearing,

tissue.³⁻⁵ Mechanical stresses are also important to chondrocyte function: the repair of damaged cartilage *in situ* is improved in moving, rather than immobilized, joints.^{6,7} Cultured chondrocytes react favorably to intermittent compression over periods less than 48 hours by increasing production of extracellular matrix.⁸⁻¹⁰ Exposure of regenerating tissue to fluid flow enhances cartilage development *in vitro* by increasing mass transfer of nutrients to and within the cell-polymer composite.¹¹⁻¹⁴ As a result of these and other observations, mechanical forces are believed to direct the differentiation of this matrix into the structure responsible for the load-bearing ability of cartilage.¹⁵

The compression/perfusion system was designed to examine the relationship between physiological levels of intermittent pressure and cartilage matrix formation. Unlike the static reactors used in past studies, the newly developed culture system, because of its capacity for medium perfusion, allows long-term study of the effects of compression on cartilage formation. Experiments to measure the uptake rates of glucose and oxygen by chondrocytes, as well as to examine the effects of different buffering systems on cell growth, were done to determine the operating conditions of the system. A five-week study of cartilage regeneration with intermittent pressurization at 500 psi demonstrates the effectiveness of the compression/perfusion system and the impact of intermittent pressure on extracellular matrix formation.

Materials and Methods

Chondrocyte Isolation

Cartilage was harvested from the stifle joints of a one week old foal (College of Veterinary Medicine, Iowa State University, Ames, IA). Chondrocytes were isolated from the tissue with type II collagenase (Worthington, Freehold, NJ) as previously described.¹⁶ The culture medium used for all experiments was Dulbecco's Modified Eagle's Medium (Gibco) supplemented with 10% fetal bovine serum (Gibco), antibiotics (10,000 U/ml penicillin and 10 mg/ml streptomycin), 10 mM HEPES, and 50 mg/mL ascorbic acid (from Sigma unless indicated otherwise). The cells were counted with a hemacytometer using the viability stain trypan blue and transferred to tissue culture flasks at a concentration of 1×10^5 cells/ml.

Polymer Matrix

A nonwoven mesh of polyglycolic acid (PGA) served as the support structure for the cells. The PGA, supplied in square pads of 1 cm x 1 cm x 5 mm thick (Albany International, Mansfield, MA), has a bulk density of 44 mg/cm³ and a porosity of 97%.

Glucose and Oxygen Consumption Rates

To determine an appropriate medium feeding strategy for the perfusion system, the consumption rates of oxygen and glucose were measured during the non-growth phase of chondrocyte culture at which most of the regeneration would be occurring. Pads of PGA (10 x 10 x 1.5 mm) were placed individually into cylindrical glass vials (20 mm i.d., 25.4 mm height) and seeded with chondrocytes (3.2×10^5 cells/pad and 2 ml medium). Each vial was capped with a rubber stopper containing an air vent (0.2 mm Acro 37 TF disposable PTFE) to

allow oxygen supply to the cells. Half of the medium in each vial was replaced every 2 to 3 days.

Between six and nine weeks of culture, glucose and oxygen concentrations and the cell density were determined for each vial, with one or two vials harvested every few days. Glucose concentrations in the culture fluid samples were determined by the Trinder assay (Sigma). Prior to determining the oxygen consumption rate, each vial was filled completely with culture medium (no gas headspace) and sealed with a stopper containing a microelectrode (Microelectrodes, Bedford, NH) which was connected to an amplifier and a volt meter. The oxygen microelectrode was calibrated immediately prior to use with air-saturated and oxygen-depleted (by N₂ sparge) water at 37°C. The viable cell density was determined using the CellTiter 96 assay (Promega, Madison, WI).

The specific oxygen and glucose uptake rates were calculated as follows:

$$\text{OUR} = -\frac{1}{X} \left(\frac{\Delta C_{\text{O}_2}}{\Delta t} \right)$$

$$\text{GUR} = -\frac{1}{X} \left(\frac{\Delta C_{\text{Gluc}}}{\Delta t} \right)$$

where OUR and GUR are the oxygen and glucose uptake rates (mol/cell-hr), X is the cell density (cells/ml), C is concentration (mol/ml), and t is time (hr).

Chondrocyte Culture in the Presence of CO₂ and HEPES Buffer

Eight 25 cm² tissue culture flasks were each seeded with 7.5×10^5 cells in 5 ml of medium with two flasks for each of four experimental groups: 10 mM HEPES buffer, no CO₂; 10 mM HEPES, 5% CO₂; no HEPES, 5% CO₂; no HEPES, no CO₂. All flasks were maintained in a 37°C CO₂ (5%) incubator; the caps were cracked open on flasks exposed to CO₂ and tightly sealed on flasks deprived of CO₂. Every three or four days, 90% of the medium was replaced. All flasks were opened for three minutes daily in a biohood to replenish the oxygen in the gas phase. The cells in each flask were released by trypsinization and counted using trypan blue after four and eleven days. After the second count, cells from the duplicate flasks of each experimental group were combined and then divided between two new flasks at a density of 7.65×10^5 cells/flask to ensure adequate time for adaptation to the culture conditions which were the same as described above. The cells were counted eight days later (day 20).

Compression/Perfusion System

A semi-continuous compression/perfusion system was specially designed and constructed to allow intermittent pressurization of developing cartilage constructs over long term durations (Fig. 1). Twelve 316 stainless steel reaction vessels (Fig. 2) are located within the incubation chamber maintained at $37 \pm 1^\circ\text{C}$ with a temperature controller (Omega) and a convective heater (Chromalox). Filtered house air flows through a pressure regulator (McMaster Carr Supply, Chicago, IL) and a series of Mac solenoid valves (Mid State Distributors, Des Moines, IA) which are electronically controlled with a programmable computer timer (Chronotrol, San Diego, CA). After being purged of air, each vessel is

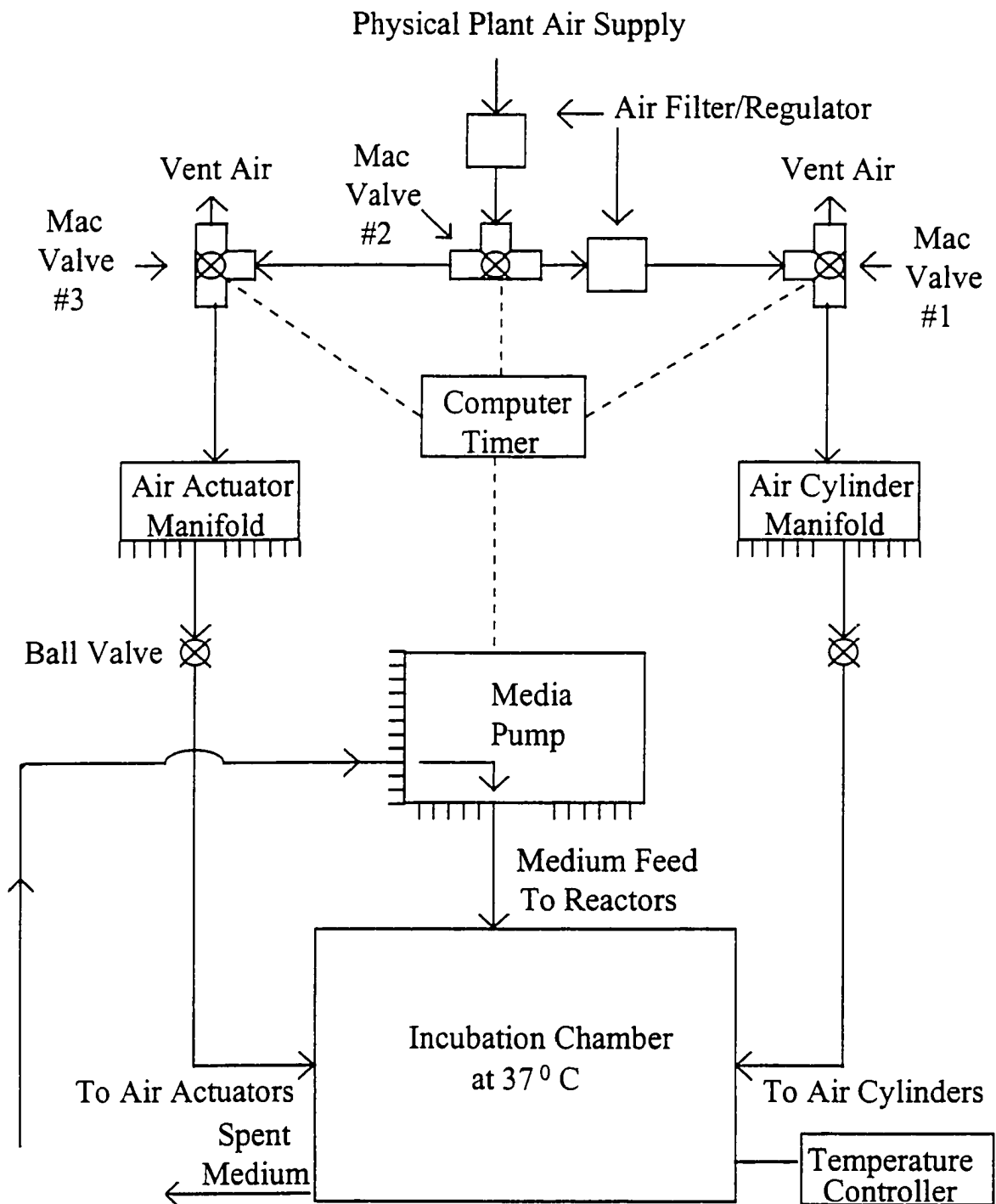


Figure 1. Diagram of the custom made compression/perfusion system for applying intermittent pressures to regenerating cartilage constructs.

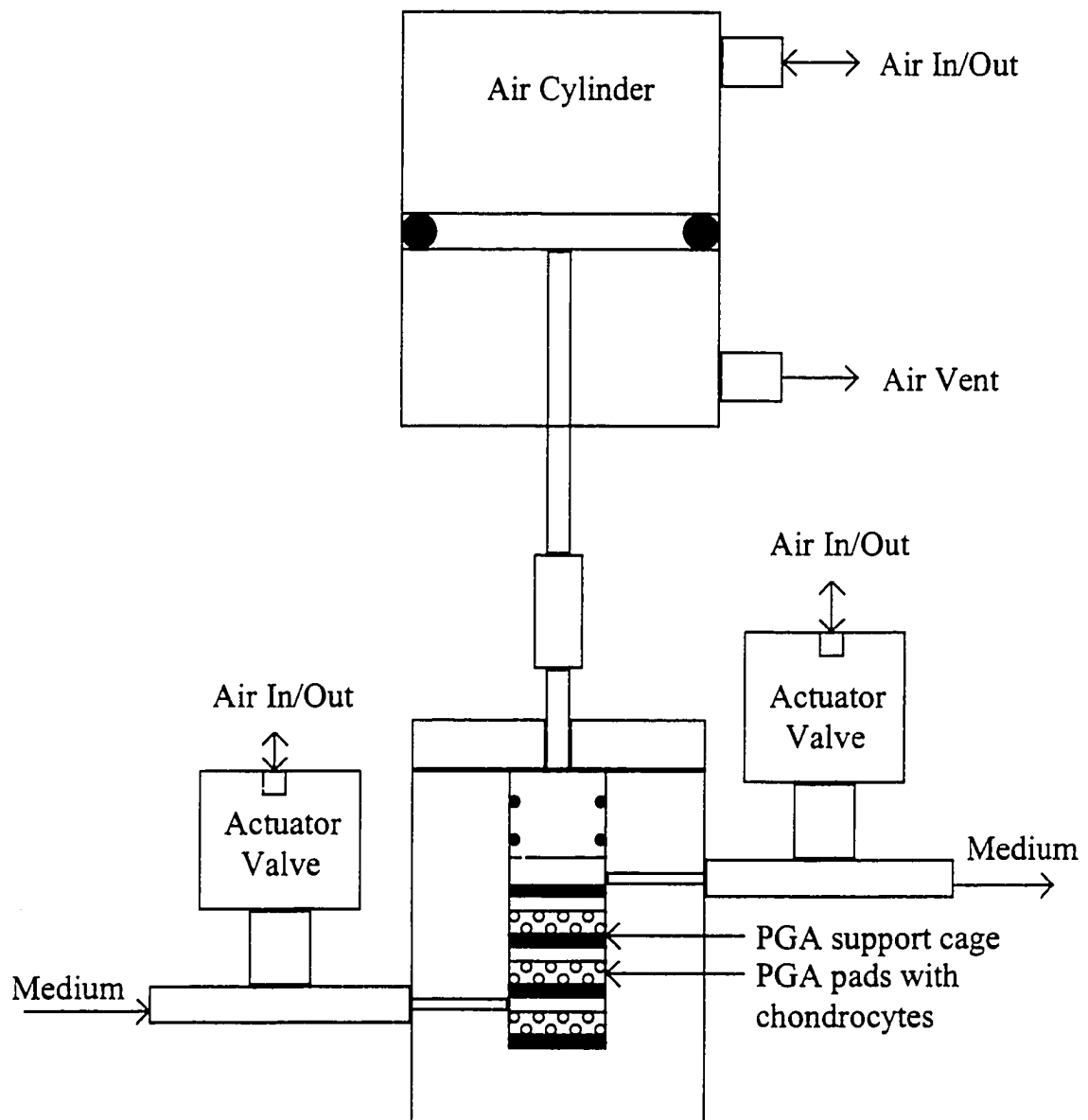


Figure 2. Expanded view of a compression/perfusion vessel and the inner support cage on which the cell-polymer constructs are cultivated for weeks to months (not drawn to scale).

pressurized by a piston, sealed with two high pressure o-rings, when air is directed to an air cylinder (Grainger, Des Moines, IA) located above each reactor. The pressure is released when the air in each air cylinder is vented to the atmosphere. The computer timer is programmed to control the duration and frequency at which each reactor is pressurized and depressurized.

The challenge in designing this system for long-term use was to permit medium perfusion in addition to providing intermittent pressure on a continuing basis, while minimizing the risk of contamination. To accomplish these objectives, air-actuated valves, designed to withstand pressures up to 3000 psi, were placed on opposite sides of each reactor. When the reactors are unpressurized and the actuator valves are opened, the timer activates a low flow multi-channel pump (Watson-Marlow) which circulates medium through each reactor and an external reservoir.

Each reactor (10 ml), which holds a cage supporting three cell-polymer composites, is attached to its own medium recirculation bottle. The bottle caps are fitted with five ports with the following functions: gas transfer with a 0.22 μm air filter (Gelman); recirculated medium inlet and outlet; fresh medium inlet; and spent medium outlet. Spent medium is bled from the recirculation bottles with a multi-channel pump (Masterflex) to a medium waste reservoir. Fresh medium is then pumped into the vessels via another multi-channel pump (Masterflex).

Cartilage Regeneration

After expansion in tissue culture flasks (three passages), the chondrocytes were collected by trypsinization, counted, and dynamically seeded onto the polymer pads.¹⁴

Dynamic cell seeding results in nearly quantitative entrapment and adherence of cells in the polymer pads as determined by the lack of any cells in the suspension or on the walls of the vessel post seeding. Before cell seeding, the polymer pads were lightly glued with Loctite Medical Adhesive (Loctite Corp.) to the three levels of each of the support cages and allowed to dry overnight. The support cages and the attached polymer pads were sterilized with ethylene oxide, followed by four washes in culture medium over a 48-hour period to remove residual ethylene oxide. Three support cages were hung from the caps of each of four 125 ml spinner flasks. Each flask contained 5×10^7 cells suspended in 125 ml culture medium, resulting in approximately 5×10^6 cells/pad. After 48 hours of slow stirring (50 rpm), the cages were removed from the spinner flasks and placed in the reaction vessels. The vessels were carefully filled with culture medium to minimize disturbance of the cell/polymer constructs. The vessels were sealed and additional medium was pumped into the vessels to remove trapped air. The feeding and pressurization regime was then initiated.

The contents of six reactors (the other six were nonpressurized controls) were pressurized intermittently (5 seconds pressurized at 500 psi and 15 seconds depressurized) for 20 minutes every four hours for 5 weeks. Prior to each pressurization, 20 ml of medium was perfused through each reactor from its recirculation vessel over a period of three minutes.

Evaluation of Regenerated Tissue

The regenerated tissue matrix formed by the chondrocytes was examined after three, four and five weeks of culture in the perfusion system. Two pressurized and two control reactors, each containing three cell constructs, were harvested each week. The three pads from each reactor were divided for analysis as follows: one half pad each for the cell, GAG

and collagen assays, one half pad for dynamic mechanical analysis, and one pad for microscopy. Native foal tissue was also assayed by the same techniques.

The number of chondrocytes per sample was determined indirectly by measuring the amount of DNA in papain digests fluorometrically using the dye Hoechst 33258 and comparing the results to a standard curve.¹⁷

A modified version of the 1,9-dimethyl-methylene blue method¹⁸ was used to quantitate the amount of GAG in the regenerated tissue. Tissue samples were desiccated, weighed, and digested for three hours to eliminate interfering proteins. The digestion was stopped with iodoacetic acid. One ml of dimethylene blue, a strong metachromatic dye, was added to 50 μ l of the digestate, mixed, and the absorbance read at 525 nm. Chondroitin sulphate b from bovine mucosa (Sigma) was used as the standard.

The compressive modulus, which is a measure of the stiffness or strength of the material, was determined from stress versus strain data obtained with a Dynamic Mechanical Analyzer (DMA-7, Perkin-Elmer) equipped with a parallel plate sample holder. The compressive modulus was determined as the slope at 1% strain.

Total collagen concentration was determined from the hydroxyproline content after acid hydrolysis of the sample followed by reaction with chloramine-T and *p*-dimethylaminobenzaldehyde.¹⁹

Data are presented as means \pm pooled standard deviations for samples analyzed within a given week. Significant differences were determined using the Student's *t* test; in Fig. 4, an asterisk indicates $p < 0.05$.

Results

Rates of Oxygen and Glucose Consumption by Chondrocytes

The specific glucose and oxygen uptake rates over the range of time tested (40 - 65 days post seeding) were relatively constant with mean values (\pm standard deviation) of 2.14×10^{-13} ($\pm 0.62 \times 10^{-13}$) and 2.05×10^{-14} ($\pm 0.44 \times 10^{-14}$) mol/cell-hr, respectively. At an average density of 2×10^7 to 3×10^7 cells/pad and three pads per vessel, and assuming medium saturated with atmospheric oxygen, a minimum flow rate of approximately 5 ml/hr is required to supply adequate oxygen to each vessel. Because the timer was set to recirculate medium after each pressurization cycle over a three-minute period as previously described, 20 ml were supplied to the reactor once every four hours.

Chondrocyte Culture in the Presence of CO₂ and HEPES Buffer

While all four groups showed no change or a drop in cell density after four days, flasks deprived of CO₂ (with or without HEPES) showed the greatest increases (average increase of 100%) in cell density by day eleven. Cultures exposed to gas containing 5% CO₂ exhibited essentially no change in cell density at eleven days. After twenty days, however, cells under all four sets of conditions showed significant increases in cell number (from 0.8×10^6 cells/ml to an average of 6.5×10^6 cells/ml), with the lowest increase found in the flasks with neither HEPES nor CO₂ (5.3×10^6 cells/ml). These results demonstrate that neither CO₂ nor HEPES buffering is necessary for chondrocyte growth as long as cells are allowed to adapt to the culture conditions.

Cell Concentrations in Tissue Constructs

Intermittent pressurization had little effect on cell density in the tissue constructs as both pressurized and non-pressurized samples showed similar patterns of growth. Cell concentrations increased in both the pressurized and control samples from weeks 3 through 5 (Fig. 3). While similar to the control at weeks 3 and 4, the cell density in the pressurized constructs was lower than the control at week 5 perhaps as a result of dilution by the increasing amount of extracellular matrix and/or because cells experiencing pressure have been shown to slow their growth.^{9,10} At 5 weeks, the control sample actually exceeded the cell density found in native foal cartilage from the stifle joint (4×10^7 to 6×10^7 cells/g tissue).

GAG Concentrations

Pressurization had a significant impact on the production of GAG in the tissue constructs (Fig. 4). The concentrations of GAG in the pressurized samples increased steadily from week 3 to week 5 and were consistently greater than those in control samples. With native foal GAG levels of 40 to 120 mg/g tissue, the pressurized samples exhibited native levels at 4 and 5 weeks. The GAG concentration in the control constructs only reached the low end of the native range at week 4, followed by a significant drop in concentration the following week.

Compressive Modulus versus GAG

The concentration of GAG and the compressive modulus were positively correlated in the pressurized (but not control) samples (Fig. 5). While multiple measures of the GAG

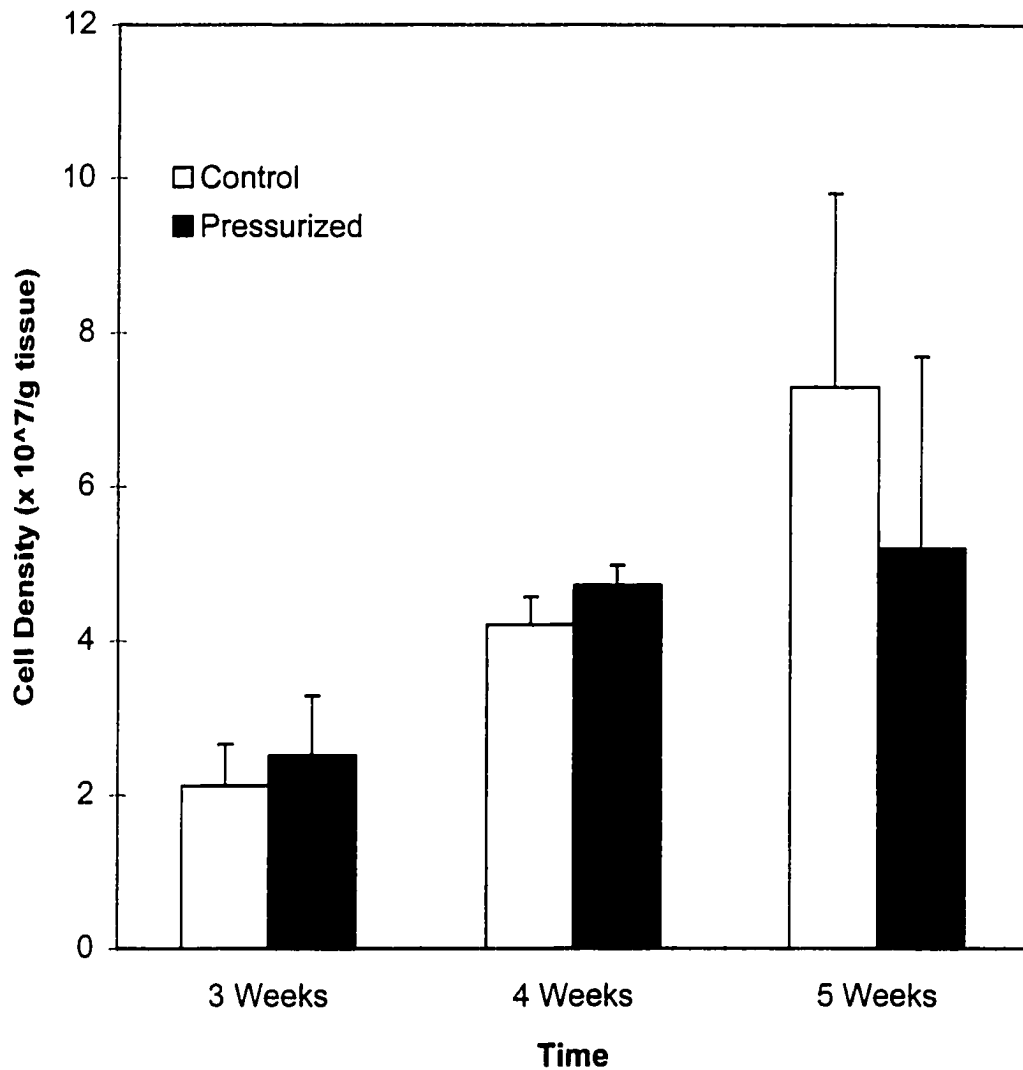


Figure 3. Cell concentrations in regenerated foal tissue with and without intermittent pressure at 500 psi. Native cell density in foal tissue is 4×10^7 to 6×10^7 cells/g wet tissue. Results are means \pm pooled SD; the cell densities did not differ significantly between pressurized and control samples at each week.

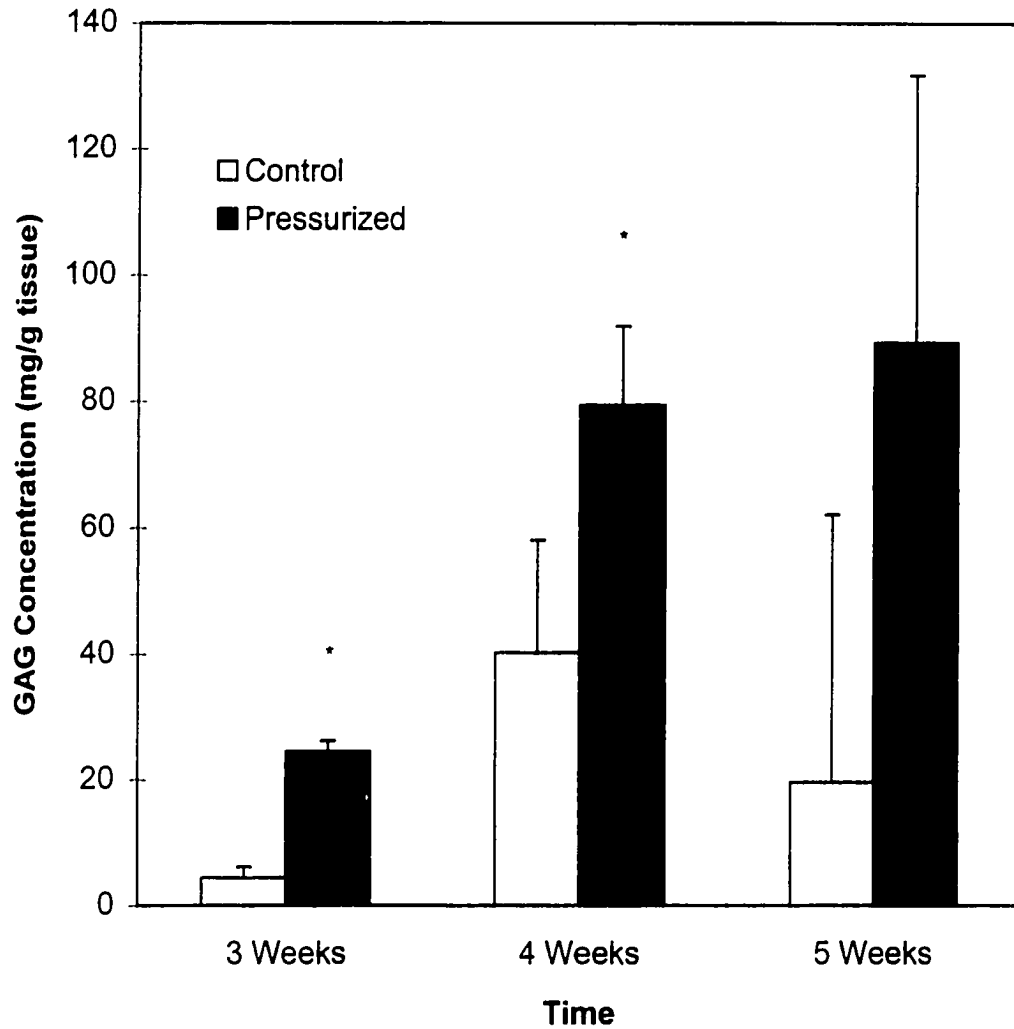


Figure 4. GAG concentrations in regenerated foal tissue with and without intermittent pressure at 500 psi. Native GAG concentration in foal tissue is 40 to 120 mg/g dry tissue. Results are means \pm pooled SD; the asterisks indicate $p < 0.05$.

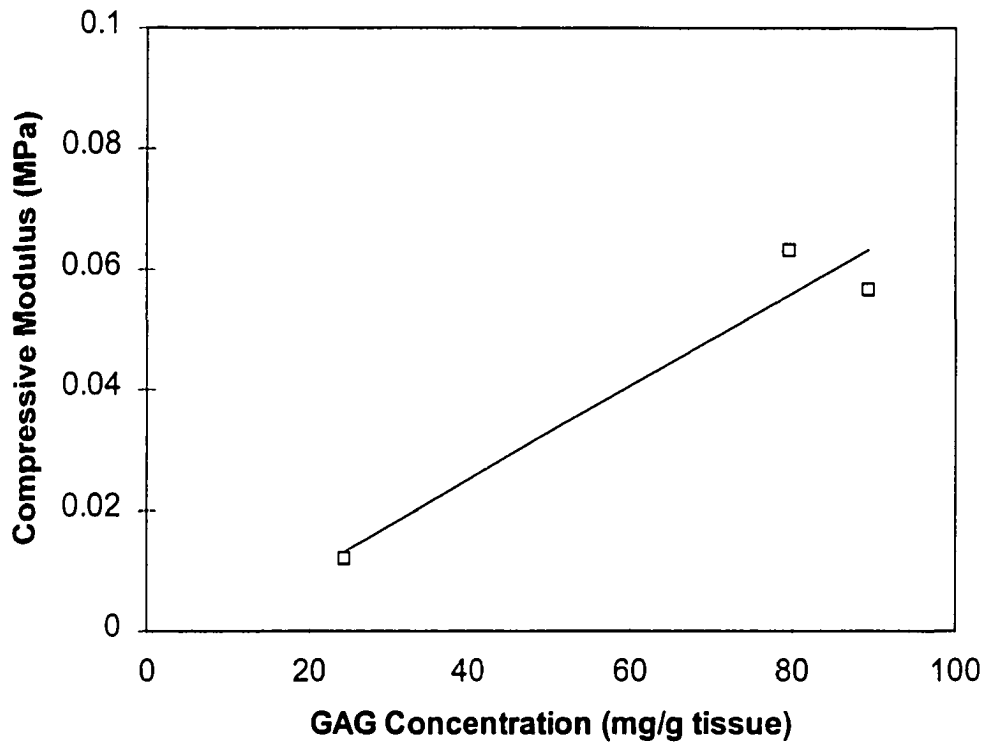


Figure 5. The compressive modulus, a measure of the construct strength, increases linearly with GAG concentration in tissue constructs subjected for 3, 4, and 5 weeks to intermittent compression at 500 psi.

content were made, the size of the sample required and the destructive nature of the compressive modulus assay allowed only a single measurement at each week. Subsequent experiments have confirmed a positive correlation between compressive modulus and the GAG content of regenerated constructs in pressurized samples (unpublished results). Because this correlation was only observed in pressurized samples, intermittent pressurization may be affecting not only the tissue composition but also its structure in some unknown manner. Even with native levels of GAG, however, the pressurized constructs yielded a modulus less than that of native tissue (0.15 - 0.30 MPa).²⁰

Collagen Concentrations

In contrast to the GAG concentrations, pressurization did not increase the production of collagen in the tissue constructs (Fig. 6). While neither regimen resulted in native foal levels of collagen (100 - 150 mg/g tissue), both pressurized and control sample concentrations increased with time and may have eventually reached native collagen levels if cultured for a longer duration.

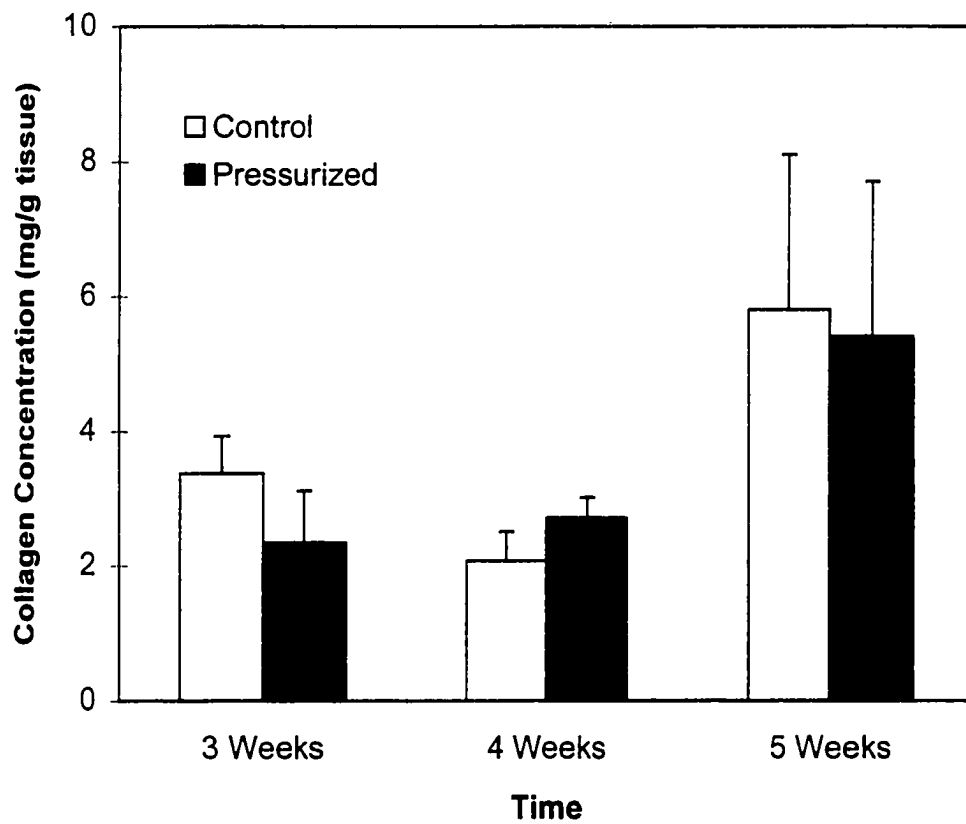


Figure 6. Collagen concentrations in regenerated foal tissue with and without intermittent pressure at 500 psi. Native collagen concentration in foal tissue is 100 to 150 mg/g wet tissue. Results are means \pm pooled SD; the collagen concentrations did not differ significantly between pressurized and control samples at each week.

Discussion

The effects of mechanical loading on the synthesis of extracellular matrix components in both isolated chondrocytes and cartilage explants has been explored but in most, if not all, cases only for time periods of 48 hours or less. Short term experiments show that, while static pressurization suppresses production,²¹ intermittent compression stimulates chondrocytes to increase synthesis of proteoglycans.¹⁰ Unless or until the pressure exceeds physiological pressures, increasing the level of intermittent compression causes an increase in the rate of proteoglycan synthesis.²²

The newly-developed compression/perfusion system was designed for long-term study of the effects of intermittent pressurization on cartilage development *in vitro*. One of the concerns in designing the system was how to best simulate the types and levels of forces experienced by chondrocytes during joint motion *in situ*. Joint motion consists of various mechanical stresses on an intermittent (but not necessarily regular) basis, with the primary loading mode in articular joints being compression. When a joint is loaded and unloaded, flow of synovial fluid past and through the tissue occurs and is responsible for load distribution, lubrication, and nutrient transport.²³ While fluid flow is necessary to the operation of the compression/perfusion system, tissue deformation, as experienced during actual joint motion, is not allowed. Compression involving deformation of regenerating tissue, especially before the extracellular matrix is fully formed, is likely to damage the porous polymer support structure and/or the cells and their extracellular matrix. Systems applying direct compressive loads and those utilizing hydrostatic pressure, however, are both valid means of studying the biological response of cartilage to mechanical stimuli.²⁴ The

choice of which to use depends on the type of questions being asked. While physical deformation may be an appropriate means of stressing explants or fully formed tissue, hydrostatic pressure is probably the best (or only) means of safely exposing regenerating tissue, particularly at the early stages of development, to compressive forces.

Hydrostatic pressurization also appears to be a more effective inducer of extracellular matrix formation in cartilage explants compared to physical compression. Matrix production in canine explants subjected to physically applied loads ranging from 0.5 to 1.2 MPa for up to 18 hours was inhibited by static compression and showed no net effect in intermittently compressed cultures.²⁵ Proteoglycan synthesis in bovine explants was enhanced, however, when subjected to hydrostatic pressures at physiological levels.²⁶ Within the hydrostatic pressure range of 2.5 to 50 MPa, matrix production was stimulated from 3.0 to ~18.0 MPa; however, as the pressure level exceeded 18.0 MPa, the rate at which proteoglycans were synthesized steadily decreased. The effects of applying hydrostatic pressure, both positive²⁷ and negative,²⁸ showed that cyclic hydrostatic pressures significantly influenced the production of extracellular matrix in explants. Newly formed proteoglycans in physically compressed explants, however, were not completely retained within the extracellular matrix.²⁹

Because the metabolism of developing cartilage has not been very thoroughly explored, it was necessary to measure the glucose and oxygen consumption rates before operating the system. While the measured glucose consumption rate (2.5×10^{-14} mol/cell-hr) is similar to that seen in other non-proliferating mammalian cells, the oxygen consumption rate (2.14×10^{-13} mol/cell-hr) is on the low end of the normal range, probably because

chondrocytes exist in an avascular tissue and possess a metabolism suited to low oxygen environments. How oxygen influences chondrocyte metabolism is not clearly understood, but articular cartilage does tolerate a fairly broad range of dissolved oxygen concentrations.²⁶ The optimum dissolved oxygen tension for cartilage regeneration, however, is not immediately clear. Whereas maximum cartilage growth and GAG synthesis occur at 21% oxygen, maximum proteoglycan aggregation occurs at 3% oxygen.³⁰ The effects of different levels of dissolved oxygen on cell metabolism exemplify the importance of a liquid-filled system at physiological pressures. Gas compression will result in increased levels of dissolved gases in the culture medium; as the applied pressure is increased, levels of dissolved gases become higher than physiological and may even become toxic. Further study is needed to determine the effects of different oxygen concentrations on cartilage development (and repair).

Also investigated was whether cartilage development would be affected by the presence or absence of dissolved carbon dioxide which is frequently used as a buffering agent with cell cultures. Because operation of a high pressure culture system is simpler without the need for CO₂ control, experiments were done to determine whether chondrocyte growth would be diminished in the absence of CO₂. Cells were grown with and without both CO₂ and HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]), an alternative buffering agent frequently used in addition to or instead of CO₂. As long as the cells were given sufficient time (two to three weeks) to adapt to the new culture conditions, the absence of CO₂ and HEPES had little effect on cell growth. The effect of CO₂ and HEPES on matrix

formation was not studied. HEPES was added to the culture medium in the present study to avoid the adaptation period, in which cell growth is slowed.

Intermittent pressurization resulted in more matrix formation than in the nonpressurized samples. While collagen concentrations generated in the compression/perfusion system were unaffected, GAG concentrations were much higher in the pressurized compared to control samples and were within the range of concentrations normally found in native foal cartilage from the stifle joint. GAG concentration was also correlated with the compressive modulus in the pressurized, but not control, samples suggesting that pressurization may also affect the tissue structure, perhaps by increasing GAG aggregation, resulting in a more organized matrix. Cell concentrations were at or near the range normally found in foal tissue but collagen concentrations were 20 to 25 times lower than native, as typically has been the case with all cartilage constructs regenerated over a five-week period.³¹

The compression/perfusion reactor was designed to support investigations of the effects of compression and fluid flow, simultaneously or separately, on the development, maintenance, and possibly degeneration of cartilaginous tissue for indefinite periods of time. Experiments to determine the effects of fluid flow and of pressure level, duration and frequency on cartilage regeneration are in progress and will be the focus of subsequent publications.

Acknowledgement

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CHAPTER 3. INCREASING EXTRACELLULAR MATRIX PRODUCTION IN REGENERATING CARTILAGE WITH INTERMITTENT PHYSIOLOGICAL PRESSURE

A paper accepted by Biotechnology and Bioengineering

Scott E. Carver and Carole A. Heath

Abstract

Isolated equine chondrocytes, from juveniles and adults, were cultured in resorbable polyglycolic acid meshes for up to five weeks with semi-continuous feeding using a custom-made system to intermittently compress the regenerating tissue. Assays of the tissue constructs indicate that intermittent compression at 500 and 1000 psi (3.44 and 6.87 MPa, respectively) stimulated the production of extracellular matrix, enhancing the rate of *de novo* chondrogenesis. Constructs derived from juvenile cells contained concentrations of extracellular matrix components at levels more like that of native tissue than did constructs derived from adult cells. With intermittent pressurization, however, even adult cells were induced to increase the production of extracellular matrix. At both levels of intermittent pressure, the concentration of sulfated glycosaminoglycan in constructs from juvenile cells was found to be up to ten times greater than concentrations in control (nonpressurized) and adult cell-derived constructs. While collagen concentrations in the 500 psi and control constructs were not significantly different for either juvenile or adult cell-derived constructs,

intermittent pressurization at 1000 psi enhanced the production of collagen, suggesting that there may be a minimum level of pressure necessary to stimulate collagen formation.

Key words: articular, cartilage, chondrocytes, regeneration, tissue

Introduction

Tissue engineering, or the development of tissues from harvested cells attached to or within a resorbable support matrix, is a relatively new strategy for the repair or replacement of damaged tissues. Transplantation of one such tissue construct, i.e., bioartificial skin, has already proven to be a successful approach for the treatment of burns and bedsores (Bell et al., 1981; Yannas et al., 1982). Another imminent application of this technology is the repair and replacement of osteoarthritic, or otherwise damaged, articular cartilage.

Articular cartilage covers the ends of all synovial joints with a one-half to five millimeters thick organized tissue and provides articulating joints with a durable, weight-distributing surface. Cartilage has well characterized histological, biochemical, and biomechanical properties, and is composed of chondrocytes, proteoglycans, collagen (primarily type II and smaller amounts of types IX and XI), and water (Mow et al., 1992). Large, highly charged, aggregating proteoglycans, localized within the collagen network, keep the tissue hydrated and resistant to compression, while cross-linked collagen fibrils give the tissue its ability to resist shear and tension. The composition and structural arrangement of these components give cartilage its mechanical properties. During normal daily function,

articular cartilage can be repeatedly subjected to forces ranging from zero at rest to several times body weight during dynamic loading (Macirowski et al., 1994).

Unlike many other tissues, cartilage is avascular, aneural, and alymphatic. While the exact mechanisms are not yet known, changes in phenotypic expression occur by transduction of mechanical signals into metabolic events and structural adaptations (Salter et al., 1980; Sah et al., 1991; Suh et al., 1995). *In vivo* studies have shown that immobilization of a joint results in degenerative changes characterized mainly by the loss of proteoglycan production (van Kampen and van de Stadt, 1987). Conversely, the *in vivo* application of mechanical force can influence the metabolic response of joint cartilage (Caterson and Lowther, 1978; Helminen et al., 1992; Kiviranta et al., 1988) and can actually improve cartilage repair following induced injury (Todhunter et al., 1993).

Most prior experiments to study the relationship between mechanical forces and cartilage development, except those of Hall et al. (1991) and Buschmann et al. (1995), were done at pressures less than physiological and/or for very short time periods. Our previous work overcame the limitation of short term experiments but the studies were still conducted at pressures lower than realized *in vivo* (Heath and Magari, 1996). Even at low pressures, however, intermittent compression resulted in the development of a qualitatively stronger matrix compared to unpressurized constructs. In the present work we report on the effects of physiological levels of intermittent compression on cartilage development over the long term, in ways not previously possible, using an intermittent pressure cell culture system with medium perfusion designed to mimic key aspects of the *in vivo* articular environment (Carver and Heath, 1998). The horse served as the model system for these experiments both because

of the intrinsic merit of and need for cartilage repair in horses and because the compositions of equine and human articular cartilage are similar (Vachon et al., 1990).

Materials and Methods

Chondrocyte Isolation

Cartilage was harvested from the stifle joints of healthy juvenile, no older than 24 months (earliest age at which the epiphyseal plate closes in the distal femur of horses (Campbell, 1977)), and adult horses (College of Veterinary Medicine, Iowa State University, Ames, IA). Chondrocytes were isolated from the tissue with type II collagenase (Worthington, Freehold, NJ) as described previously (Klagsbrun, 1979). The culture medium used for all experiments was Dulbecco's Modified Eagle's Medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco), antibiotics (10,000 U/ml penicillin and 10 mg/ml streptomycin, Gibco), 10 mM HEPES (Sigma Chemical, St. Louis, MO), and 50 µg/mL ascorbic acid (Sigma). The cells were counted with a hemacytometer using the viability stain Trypan Blue (Sigma) and cultured in flasks (Corning, Corning, NY) at a concentration of 1×10^5 cells/ml.

Compression/Perfusion System

To conduct these studies, we designed and constructed a semi-continuous perfusion system capable of administering intermittent compression to cartilage constructs (Carver and Heath, 1998). Briefly, house air enters a pressure regulator and is adjusted to the appropriate pressure (up to 80 psi). A three-way solenoid valve, located between the pressure regulator and the air manifold, distributes pressurized air to each of the air cylinders (one per culture

vessel). By switching the solenoid valve, air is either forced into or out of the manifold and the pistons used to pressurize the twelve culture chambers. Since the diameter of each air cylinder is five times that of each chamber piston, the pressure in each culture vessel is 25 times the regulated pressure, allowing pressurization of the reactors (containing the cell-polymer composites) up to 2000 psi (~13.5 MPa), which is near the high end of pressures normally experienced by the human hip (Macirowski et al., 1994). Normal, daily contact stresses on human joints range from 3 to 10 MPa (Mow et al., 1992). The actual pressure in the reaction vessels was verified, and found to be close to the calculated pressure, by attaching a pressure gauge to the medium inlet port (to which an air-actuated valve is normally attached as described below) in the vessel.

Air pressure was also used to open and close actuated valves (Omaha Valve and Fitting; Omaha, NE) located on opposite sides of each reactor to allow medium perfusion. When the intermittent pressure was off and thus the pressure in the chambers was approximately atmospheric, the valves were open. Medium was then pumped into and out of the reactors via a twelve channel pump (Lab Research Products; Lincoln, NE) from external reservoirs. The reactors were housed inside an incubation chamber which was maintained at 37°C. Inside each reactor was a three-tiered support cage to which three polymer pads were adhered.

The vessels were kept completely filled with culture medium for the duration of each experiment. Gas compression would have resulted in an unreasonably large deflection of the piston to achieve pressurization and a high, potentially toxic, dissolved oxygen concentration

in the culture medium. As a result of the very low compressibility of culture medium, the piston moved very little during the compression.

Polymer Preparation

The PGA nonwoven mesh (97% void volume, 13 μm diameter fibers) was supplied in square pads of 1 cm x 1 cm x 5 mm thick (Albany International, Mansfield, MA). According to the manufacturer, the PGA is completely resorbed over a period of 120 days and loses its strength by about 30 to 40 days *in vitro*. Before cell seeding, the polymer pads were lightly glued with Loctite Medical Adhesive (Loctite Corp.) to the three levels of each support cage and allowed to dry overnight. The support cages and the attached polymer pads were sterilized with ethylene oxide (EtO), followed by four washes in culture medium over a 48-hour period to remove residual EtO and to test for sterility.

Cartilage Regeneration

After expansion in tissue culture flasks (approximately three passages), the chondrocytes were collected by trypsinization, counted, and dynamically seeded into PGA pads at a density of 5×10^6 to 7.5×10^6 cells/pad as previously described (Carver and Heath, 1998). Dynamic cell seeding results in a more uniform distribution of cells throughout the polymer pad (Vunjak-Novakovic et al., 1996). The support cages were placed in the perfusion chambers, which were carefully filled with culture medium to minimize disturbance of the cell/polymer constructs. The vessels were sealed and additional medium was pumped into the vessels to remove trapped air. The constructs were maintained continuously in the culture vessels until harvest.

Four different experiments were conducted to examine the effects of intermittent pressurization level and age of the donor tissue on cartilage regeneration by the isolated chondrocytes. Constructs containing chondrocytes from both juvenile and adult horses were each subjected to intermittent pressures of 500 and 1000 psi. In each experiment, half of the reactors (six) were pressurized and the other half served as nonpressurized controls; all other culture conditions were the same. The intermittent pressure regime (five seconds pressurized and fifteen seconds depressurized) was applied for 20 minutes every four hours for five weeks; prior to pressurization each reactor was perfused with 20 ml of medium from its recirculation vessel over a period of three minutes.

Evaluation of Regenerated Tissue

The *de novo* tissue matrix formed by the chondrocytes was examined at three, four and five weeks of culture in the compression/perfusion system. In most cases, two pressurized and two control reactors were sampled each week giving duplicate samples for analysis. The three constructs from each reactor were typically divided as follows: one half construct for each of the cell, GAG and collagen assays, one half construct for dynamic mechanical analysis, and one construct for microscopy. Native tissue from juvenile and adult horses was also assayed.

Cells. The number of chondrocytes per sample were determined in quadruplicate from a standard curve by fluorometrically measuring the amount of DNA in papain digests using the dye Hoechst 33258 (Kim et al., 1988). Calf thymus DNA was used as the standard. A DNA concentration of 7.7 pg/chondrocyte was used to calculate cell numbers per construct.

GAG. A modified version of the method of Farndale et al. (1986) was used to measure in quadruplicate the amount of GAG in the regenerated tissues. Tissue samples were desiccated, weighed, and digested for three hours. The digestion was stopped with iodacetic acid. One ml of dimethylene blue was added to 50 μ l of the digestate, mixed, and the absorbance read at 525 nm. Chondroitin sulphate b from bovine mucosa (Sigma) was used as the standard.

Collagen. Total collagen content was determined in triplicate from the hydroxyproline concentration. The samples were hydrolyzed with 6 N HCl for 18 hours at 115°C and then reacted with chloramine-T and *p*-dimethylaminobenzaldehyde (Woessner, 1961).

Transmission Electron Microscopy (TEM). The collagen fiber density in constructs was qualitatively examined by TEM. Samples were washed and fixed in 2% paraformaldehyde/3% glutaraldehyde in a 0.1 M PBS buffer at 4 °C for 48 hours. After three washes, the samples were rinsed and immersed in 1 % osmium tetroxide in the same buffer for two hours at room temperature. After rinsing, the samples were stained with 2% aqueous uranyl acetate overnight, dehydrated through a graded alcohol series, and placed in acetone. The pieces were embedded in a resin consisting of succinic anhydride, nadic methyl anhydride, Embed epoxy resin and 2,4,6-tri(dimethylaminomethyl)phenyl. The resin was polymerized by placing the samples in a 60°C oven for two days. The resin blocks were cut into 50-80 nm thin sections with a diamond knife, placed on copper slides, and stained with lead citrate and uranyl acetate. The samples were viewed on a JEOL 1200 EX II scanning transmission electron microscope and the images were photographed with Kodak 50-163.

Statistics. Data are presented as means \pm 95% confidence with pooled standard deviations for samples analyzed within a given week.

Results

Cell Concentration

With a few exceptions at 1000 psi, cell concentrations did not differ significantly between pressurized and control samples in both the juvenile and adult tissue constructs (Table I). At four and five weeks, cell concentrations in all pressurized constructs were less than or equal to the concentrations in control constructs; pressurization has also been shown to reduce cell proliferation in explants and cell cultures (Palmoski and Brandt, 1984; van Kampen et al., 1985). Native juvenile and adult cell densities were found to be 4×10^7 to 6×10^7 cells/g tissue and 2×10^7 to 4×10^7 cells/g tissue, respectively. All conditions led to constructs with cell densities near or within the normal range at all sample times.

Table I. Concentration of chondrocytes (cells/g tissue $\times 10^{-7}$) in the cell/polymer constructs.

Week	Juvenile Control	Juvenile 500 psi	Juvenile 1000 psi	Adult Control	Adult 500 psi	Adult 1000 psi
3	2.21 ± 1.15	2.52 ± 1.99	3.98 ± 1.99	4.62 ± 1.15	6.05 ± 1.40	1.27 ± 1.99
4	4.16 ± 1.01	4.70 ± 1.01	3.91 ± 1.43	3.61 ± 1.01	3.46 ± 1.01	3.49 ± 1.43
5	5.37 ± 1.52	5.20 ± 1.87	1.33 ± 2.64	4.97 ± 1.87	2.61 ± 1.99	1.59 ± 2.64

Sulfated Glycosaminoglycan Concentration

Constructs which were intermittently compressed exhibited greater concentrations of GAG than the non-pressurized controls at both pressures for the juvenile (Fig. 1a) and at 500 psi for the adult (Fig. 1b) cells. For the juvenile cells, the concentration of GAG in the constructs was influenced by the level of applied intermittent pressure, with the higher pressure resulting in higher GAG concentrations (Fig. 1a). At 1000 psi, GAG concentrations in the pressurized and control adult constructs were not significantly different. At both pressures, GAG concentrations in the adult-derived constructs were consistently lower than those produced by juvenile chondrocytes. While tissues developed from adult chondrocytes

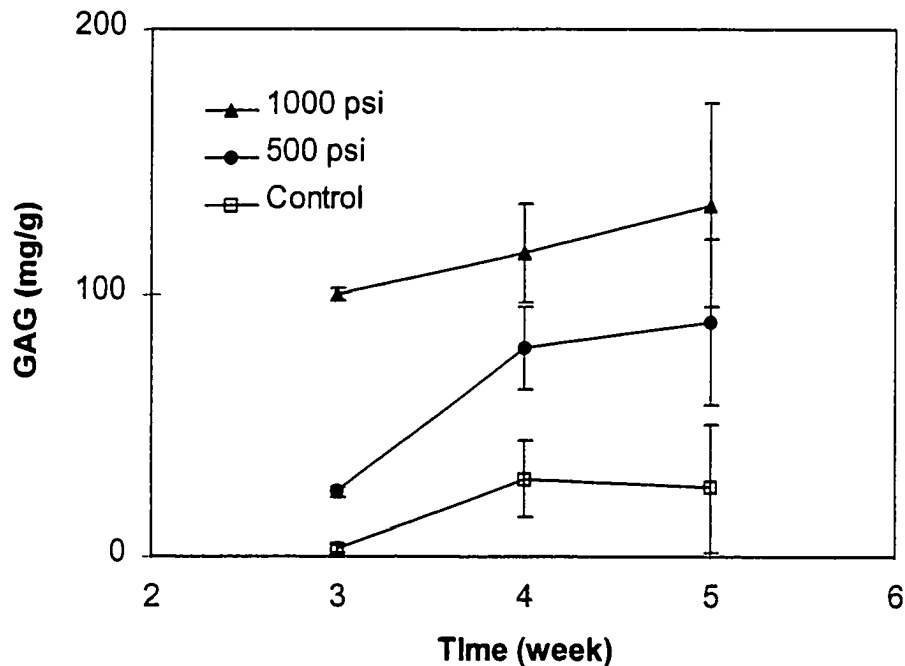


Figure 1a. The GAG concentration in tissue constructs from juvenile cells was increased by intermittent compression at 500 and 1000 psi. Note the difference in scale between the juvenile and adult (Fig. 1b) GAG concentrations.

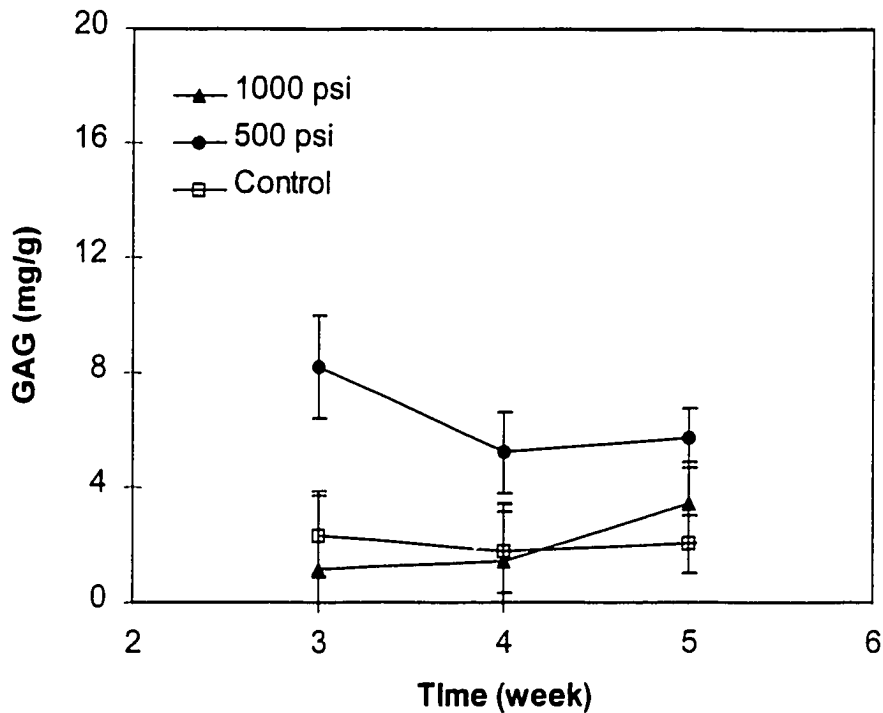


Figure 1b. Intermittent compression at 500, but not 1000, psi stimulated GAG production in tissue constructs from adult cells.

did not reach native concentrations of GAG (80 to 120 mg/g tissue) under the given growth conditions and length of time cultured, both juvenile experiments resulted in constructs with GAG concentrations within the normal range for juvenile tissue (40 to 120 mg/g tissue) at five weeks.

The production of GAG on a per cell basis was calculated to determine whether the differences in GAG concentrations between the juvenile and adult constructs resulted, in part or in total, from differences in cell density (Table II). Although the adult cells produced essentially no GAG between weeks three and five, the specific average production of GAG

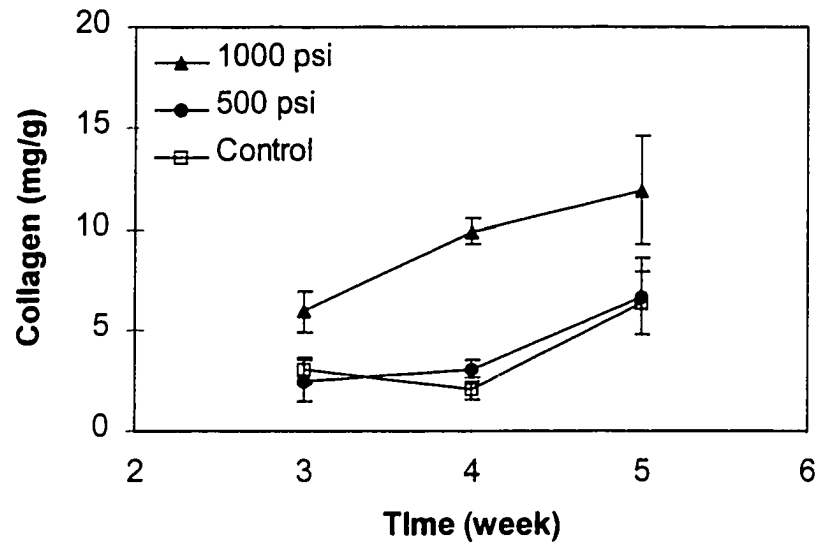
Table II. Average specific production of extracellular matrix between weeks 3 and 5.

Construct	GAG (mg/10 ⁷ cells)	Collagen (mg/10 ⁷ cells)
Juvenile Control	0.09	0.80
Juvenile 500 psi	0.45	0.74
Juvenile 1000 psi	1.94	1.95
Adult Control	0.01	-0.16
Adult 500 psi	-0.03	0.47
Adult 1000 psi	0.11	2.65

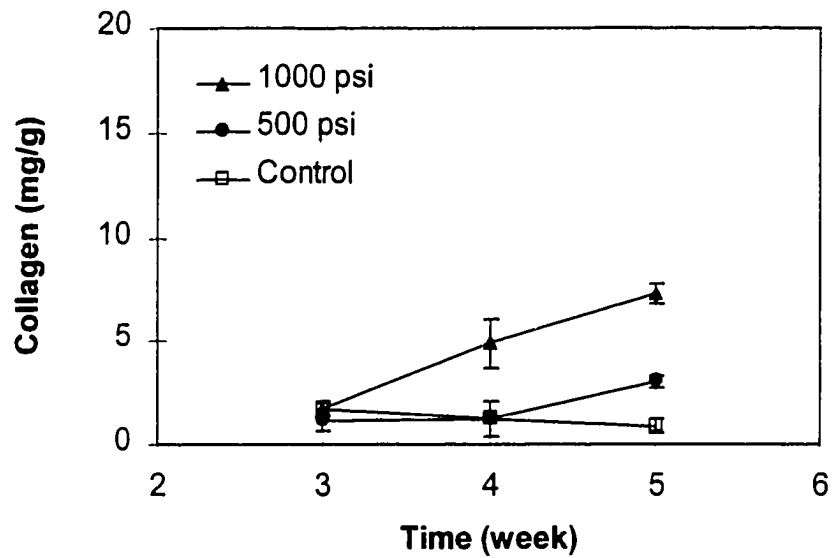
(mg/10⁷ cells) by juvenile cells increased twenty-fold from the control condition to intermittent pressurization at 1000 psi, indicating that differences in cell density were not responsible for the higher GAG concentration in juvenile constructs. Instead, the ability of intermittent pressurization to stimulate GAG production in juvenile, but not adult, cells is confirmed. Increased cell density, therefore, would not increase the GAG content to native levels in adult constructs, even with intermittent pressurization.

Collagen Concentration

The level of applied pressure was particularly important in the production of collagen in the constructs. With both juvenile and adult cells, intermittent pressurization at 1000 psi resulted in greater collagen concentrations compared to those at 500 psi and the unpressurized controls (Fig. 2). Pressurization at 500 psi had little effect on collagen



A



B

Figure 2. The collagen concentration in tissue constructs from juvenile (a) and adult (b) donor cells is increased by intermittent compression at 1000, but not 500, psi suggesting that there may be a minimum pressure to evoke a stimulatory response.

concentration as the constructs did not contain any more collagen than nonpressurized controls (except at five weeks in adult constructs). While all regenerated constructs had collagen concentrations well below native levels (100 to 150 mg/g tissue and 120 to 180 mg/g tissue for juvenile and adult cartilage, respectively), juvenile constructs contained approximately twice as much collagen as adult constructs cultured under similar conditions. The stimulative effect of intermittent pressure is also demonstrated on a per cell basis (Table II). In the absence of pressure, juvenile cells produce a low level of collagen, while adult cells produce essentially none. With intermittent pressure, however, both juvenile and adult cells can be induced to produce higher levels of collagen, especially at 1000 psi. On a per cell basis, adult cells are as good as, or better than, juvenile cells as producers of collagen. With similar cell densities, adult constructs should be able to achieve collagen concentrations equivalent to that in juvenile constructs if stimulated with intermittent pressurization.

Microscopy

Qualitative analysis of the extracellular matrix of the constructs regenerated from adult chondrocytes (500 psi intermittent compression and control) was made by transmission electron microscopy (Fig. 3). TEM sections showed an increase in collagen density in pressurized samples from three to seven weeks (Fig. 3 a,b) and between intermittently pressurized and control samples at seven weeks (Fig. 3 b,c). The banding pattern indicative of Type II collagen is apparent in Fig. 3b.

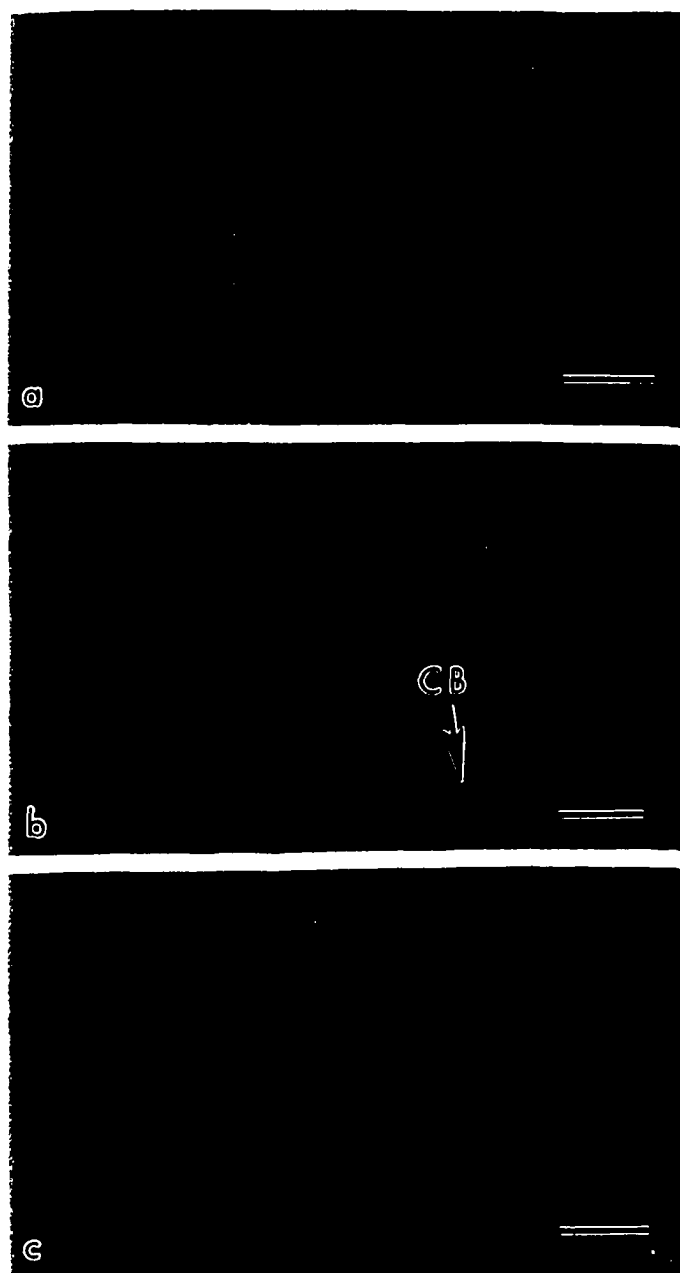


Figure 3. Transmission electron microscopy sections, stained with lead citrate/uranyl acetate and photographed at 60,000X, show the collagen fibrils in tissue constructs from adult equine chondrocytes. The isolated cells were cultured in a PGA mesh with and without (control) intermittent pressurization at 500 psi. Pressurized constructs illustrate increased collagen fibril density between three (a) and seven (b) weeks; the nonpressurized control at seven weeks (c) shows little collagen. The thick banded (CB) structure indicative of type II collagen is visible at seven weeks with pressure (b). Each bar represents 0.1 μm .

Discussion

Many studies have been conducted to investigate the effects of static or dynamic mechanical force on cartilage explants or chondrocytes in culture but not for tissue constructs and, in most cases, only for short periods of time (48 hours or less). While static compressive loading inhibits GAG synthesis (Jones et al., 1982; Schneiderman et al., 1986; Gray et al., 1988), cyclical loading over a short time period inhibits cell division (van Kampen et al., 1985; Veldhuijzen et al., 1987) and promotes GAG synthesis and overall matrix production (Palmoski and Brandt, 1984; van Kampen et al., 1985; Veldhuijzen et al., 1987; Burton-Wurster et al., 1993; Bacharach et al., 1995; Farquhar et al., 1996; Torzilli et al., 1997). There appear to be limits, both for magnitude and frequency of the pressure cycle, outside of which these effects may be detrimental (Burton-Wurster et al., 1993; Parkkinen et al., 1993; Bacharach et al., 1995; Farquhar et al., 1996), and there is evidence of a "rebound" effect with increased cellular metabolism following release of pressure (Lippiello et al., 1985). Generally speaking, application of stresses below normal physiological levels appears to stimulate catabolic activity and within the physiological range leads to maintenance of explants (Burger et al., 1991). Stresses higher than physiological are likely to result in tissue damage (Farquhar et al., 1996).

The aim of this study was to investigate the effects of physiological levels of intermittent compression on the development of cell/polymer constructs, using cells from both juvenile and adult animals, over a period of several weeks. These experiments were made possible by a specially designed system implementing semi-continuous medium

perfusion and intermittent hydrostatic pressurization (Carver and Heath, 1998). The system is capable of applying different levels, durations, and frequencies of compression as well as different rates of fluid flow to developing tissues inside the culture vessels. While other systems have been developed for long term culture of cartilage constructs (Dunkelman et al., 1995; Bursac et al., 1996; Vunjak-Novakovic et al., 1996; Freed and Vunjak-Novakovic, 1997), none have also incorporated the ability to intermittently pressurize the constructs during development.

While there was some variation in the results, intermittent pressurization reduced cell proliferation and increased the amount of secreted matrix (GAG and collagen) in the tissue constructs. Although the cell concentrations were at or near native tissue levels, intermittent compression typically resulted in lower cell densities than the unpressurized controls, especially in the week five constructs. The lower cell density with pressure is consistent with short-term experiments and suggests that mechanically stressed cells may direct their anabolic efforts towards production and maintenance of matrix rather than cell proliferation (van Kampen et al., 1985; Veldhuijzen et al., 1987).

The concentration of GAG in the tissue constructs was affected both by the level of pressurization and by the age of the donor cells. In constructs derived from juvenile cells, intermittent compression resulted in a greater GAG concentration, with higher concentrations exhibited at the higher level of pressure. This observation is consistent with animal studies showing higher levels of GAG in areas of frequently loaded cartilage (Caterson and Lowther, 1978; Kiviranta et al., 1988; Salter et al., 1980). Juvenile cells produced higher concentrations and amounts per cell of GAG than adult cells, resulting in native levels of

GAG in the juvenile constructs. All adult constructs had GAG concentrations well below native levels indicating that, while adult cells may respond to pressure with a small increase in GAG production, they are poor candidates for *de novo* chondrogenesis. The concentration of proteoglycans is particularly important since they keep the tissue hydrated and their loss has been shown to be one of the first events in cartilage degeneration (Cheung et al., 1978). Because they have half-lives on the order of weeks or less (Hendrickson et al., 1994), proteoglycans must continuously be produced to maintain tissue structure and function.

In contrast to the increase in GAG concentration at both levels of pressure, collagen concentration was no different between control constructs and those pressurized at 500 psi. At an intermittent pressure of 1000 psi, however, collagen concentrations were significantly greater than the control in both adult and juvenile constructs, suggesting that there may be a minimum level of pressure needed to stimulate collagen formation. While neither adult nor juvenile constructs reached native levels, collagen concentrations in the juvenile constructs were significantly higher than those in the adult constructs. This difference in collagen content, however, resulted at least in part from differences in cell density between the juvenile and adult constructs since, on a per cell basis, adult cells can produce similar amounts of collagen when stimulated by intermittent pressure. The increase in the rate of collagen production as a function of time suggests that longer times are needed to achieve the levels of collagen found in native tissues. Higher levels of intermittent pressure may also result in greater stimulation of collagen production. Other factors, i.e., addition of transforming growth factor β to the culture medium (Zimber et al., 1995) and use of collagen

scaffolds (Grande et al., 1997), increase the production of type II collagen in tissue constructs and could be implemented with intermittent compression.

Despite the increase in matrix production with intermittent pressure, adult cells are much less effective in *de novo* chondrogenesis than juvenile cells primarily because of their limited ability to produce native levels of GAG. This result is consistent with earlier work demonstrating that chondrocytes isolated from immature rather than mature donors produce an improved cartilage surface (Chesterman and Smith, 1968; Bentley and Greer, 1971).

From a practical standpoint, development of replacement tissue or injection of chondrocytes into articular cartilage lesions might be more effective if the recipient's own cells could be used since the possibilities of rejection and virus transmission would be eliminated. Cells from young donors, however, proliferate more rapidly and are much better producers of extracellular matrix. Fortunately, articular cartilage is avascular and alymphatic, and its cell density is one of the lowest in the body, greatly reducing the likelihood of rejection.

Constructs made from a combination of autologous and young donor cells might provide a suitable compromise.

How the cells sense hydrostatic pressure and alter their metabolic activities is still unclear. While there is a large body of evidence to support a correlation between cell shape and the expressed phenotype (e.g., Glowacki et al., 1983; Newman and Watt, 1988), our work, as well as that of others (Horton and Hassel, 1986; Benya et al., 1988; Brown and Benya, 1988; Mallein-Gerin et al., 1990), suggests that cell shape is only one of several factors influencing cellular activity. Physical deformation of chondrocytes in explants does cause changes in cell shape and volume as well as in their phenotypic expression

(Buschmann et al., 1996). Cells in this study, however, should not have experienced a change in shape because the hydrodynamic compression was isotropic. Because of the pressure differential across the cell membrane, the cells may have experienced instead a small change in volume which could have affected the structural arrangement of the cytoskeleton. Changes in cytoskeletal structure may be more directly responsible for changes in phenotypic expression than cell shape or volume alone (Brown and Benya, 1988; Mallein-Gerin et al., 1990; Wang et al., 1993). The presence of extracellular matrix also appears to be important as stimulation of biosynthesis was enhanced following dynamic compression of chondrocytes immobilized in agarose gel when more matrix was present around the cells (Buschmann et al., 1995). Additional study is needed to determine how these factors and others influence phenotypic expression of loaded cartilage.

Although the cartilage constructs developed in this study exhibited near native concentrations of cells and GAG and higher concentrations of collagen than we have seen in the past, the constructs were smaller in size and mass than those produced by other investigators (Freed and Vunjak-Novakovic, 1997). As has already been demonstrated (Bursac et al., 1996; Vunjak-Novakovic et al., 1996; Freed et al., 1993, 1994 a,b), small constructs are most likely a result of mass transfer limitation. In the experiments described in this paper, fluid flow in the chambers containing the constructs only occurred for 18 minutes each day (6.67 ml/min) and was not turbulent; the fluid surrounding the constructs was stagnant at all other times. These results confirm that convective transport is a critical component of nutrient transfer especially in the early stages of culture when the constructs are still very porous (Bursac et al., 1996). Experiments are in progress to determine whether

fluid flow for longer periods and at higher velocity will increase the accessibility of nutrients to the inner regions of the constructs and result in larger, stronger tissues.

Conclusions

Using the custom-made compression/perfusion system, intermittent pressurization of cell-polymer constructs resulted in an increased level of *de novo* chondrogenesis compared to nonpressurized controls. Intermittent pressure at physiological levels increased extracellular matrix formation for both adult and juvenile chondrocytes but with constructs from juvenile cells exhibiting extracellular matrix compositions more like that of native tissue. The use of cells from young animals appears to be critical for extracellular matrix formation and the successful development of tissue constructs *in vitro*. Sulfated glycosaminoglycan concentrations increased in juvenile constructs as the level of intermittent pressure increased and were within the range found in native tissue. Collagen concentration levels were not affected at the lower intermittent pressure (500 psi). At 1000 psi, however, collagen synthesis increased, suggesting that there may be a minimum intermittent pressure needed to stimulate collagen production.

Acknowledgments

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CHAPTER 4. THE INFLUENCE OF PRESSURIZATION, FLUID FLOW, AND MIXING ON THE REGENERATIVE PROPERTIES OF ARTICULAR CHONDROCYTES

A paper submitted to Biotechnology and Bioengineering

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Abstract

Equine articular chondrocytes, embedded within a polyglycolic acid nonwoven mesh, were cultured with various combinations of pressurization, fluid flow, and mixing to examine the effects of different physical stimuli on *de novo* chondrogenesis. The cell/polymer constructs were cultured first in 125 ml spinner flasks for one, two, or four weeks and then in a perfusion system with physiological intermittent pressure for a total of up to six weeks. Additional constructs were either cultured for all six weeks in the spinner flasks or for one week in spinners followed by five weeks in the perfusion system without intermittent compression. Tissue constructs cultivated for two or four weeks in spinner flasks followed by perfusion with intermittent pressurization had significantly higher concentrations of both sulfated glycosaminoglycan and collagen than constructs cultured entirely in spinners or almost entirely in the compression/perfusion system. Initial cultivation in the spinner flasks, with turbulent mixing, enhanced both cell attachment and early development of the extracellular matrix. Subsequent culture with perfusion and intermittent compression appeared to accelerate matrix formation. While the correlation was much stronger in the

pressurized constructs, the compressive modulus was directly proportional to the concentration of sulfated glycosaminoglycan in all physically stressed constructs. Constructs which were not stressed beyond the one week seeding period lost mechanical integrity upon harvest suggesting that administration of physical forces, particularly compression, to tissue constructs during their development may be an important determinant of their ultimate biomechanical functionality.

Keywords: cartilage, chondrocytes, mixing, pressure, tissue

Introduction

Osteoarthritis, in which the hyaline cartilage of the articulating joints is irreversibly degraded, affects more people in the United States than all other forms of arthritis combined (Brewerton, 1992). In an effort to cure this often debilitating disease, many researchers have investigated using cell-based therapies as an alternative to total joint replacements. If detected early enough, osteoarthritic degradation can be arrested with localized cell injections (Brittberg et al., 1994). If the defect becomes too large, however, the cells require a structured framework at the defect site until they can create their own extracellular matrix (ECM) for support. Chondrocytes, the only cell type found in articular cartilage, possess the ability to produce the two main organic components normally comprising the ECM, namely type II collagen and proteoglycan.

In order for articular chondrocytes to produce the appropriate types and concentrations of the ECM components, they must be cultured in an environment that mimics

certain aspects of the *in vivo* system. Culture in a three-dimensional environment is needed for production of type II collagen (Benya and Shaffer, 1982). Various biocompatible materials, primarily resorbable polymers, have been used to provide articular chondrocytes with a three-dimensional structure (Klompmaaker et al., 1992; Freed et al., 1993; Kim et al., 1994; Grande et al., 1997). When the cell/polymer constructs are of the same dimensions as *in vivo* tissue, however, they must be cultured in a well-mixed environment to overcome diffusion limitations of nutrients to cells in the center of the constructs (Vunjak-Novakovic et al., 1996). While mixed systems support growth of tissue constructs with many of the same biochemical characteristics as articular cartilage, additional forces may be needed for the development of a mechanically functional biological graft.

In vivo, articular cartilage is frequently loaded as the joint performs its normal functions. When pressurized dynamically, chondrocytes increase production of proteoglycan (Veldhuijzen et al., 1987; Parkkinen et al., 1993; Burton-Wurster et al., 1993), which plays the important role of maintaining tissue hydration and resistance to compression. The benefits of applying dynamic forces at physiological levels (Torzilli et al., 1997), over long-term durations (Heath and Magari, 1996), and to chondrocytes cultured in a three dimensional environment (Buschmann et al., 1995) have been demonstrated; however, a system which combines all of these conditions has only recently been developed (Carver and Heath, 1998a). With this system, physiological levels of intermittent pressure enhanced the production of ECM in cartilage constructs (Carver and Heath, 1998b). The tissue constructs contained native levels of cells and sulfated glycosaminoglycans (GAG) and possessed higher concentrations of collagen than without pressurization. Without convection, however,

mass transfer limitations resulted in tissue constructs that were smaller in size and mass than have been developed in mixed systems (Freed and Vunjak-Novakovic, 1997). The purpose of this study was to determine whether combination of the separately-advantageous conditions of mixing/fluid flow and intermittent pressurization would synergistically enhance the development of cartilaginous tissue constructs from isolated equine chondrocytes.

Materials and Methods

Chondrocyte Isolation

Articular cartilage was surgically excised from the stifle joint of healthy foals (juvenile horses) no older than one month (College of Veterinary Medicine, Iowa State University, Ames, IA). The tissue was enzymatically digested using type II collagenase as previously described (Klagsbrun, 1979) and the digestate was collected in sterile centrifuge tubes. After two washes in 0.1 M phosphate buffered saline, a cell pellet was formed and resuspended in culture medium. The culture medium used for all experiments was Dulbecco's Modified Eagle's Medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco), antibiotics (10,000 U/ml penicillin and 10 mg/ml streptomycin; Gibco), 10 mM HEPES (Sigma Chemical, St. Louis, MO), and 50 µg/ml ascorbic acid (Sigma). The cells were counted with a hemacytometer using Trypan Blue (Sigma) to test for viability and cultured in 225 cm² tissue culture flasks (Corning, Corning, NY) at a concentration of 1×10^5 cells/ml.

Polyglycolic Acid Substrate Preparation

The polyglycolic acid (PGA) nonwoven mesh (97% void volume, 13 μ m diameter fibers) was supplied in square pads measuring 1 cm x 1 cm x 0.5 cm thick (Albany International, Mansfield, MA). According to the manufacturer, PGA is completely resorbed over a period of 120 days and loses its strength by about 30 to 40 days *in vitro*. Prior to cell seeding, the polymer pads were placed in screen tissue baskets (Ted Pella, Redding, CA) which were modified to allow additional medium flow through the endcaps. The support baskets and the enclosed polymer pads were sterilized with ethylene oxide (EtO), oven dried, and washed with culture medium to remove residual EtO and to pre-wet the polymer pads.

Mixed Cultures

After three passages, the chondrocytes were trypsinized, collected in culture medium, and placed in 125 ml spinner flasks each containing six tissue baskets resting on the bottom of the vessel. The chondrocytes were dynamically seeded into the pads at a density of approximately 4×10^6 cells/pad as previously described (Vunjak-Novakovic et al., 1996). The flasks were stirred at approximately 50 rpms and were maintained in incubators at 37°C and 5% CO₂. Seventy-five percent of the culture medium was replaced weekly until the tissue baskets were harvested or moved to the compression/perfusion system.

Pressurized Cultures

At one, two or four weeks post seeding, cell/polymer constructs were transferred from spinner flasks to the compression/perfusion system which allows the application of physiological levels of intermittent hydrostatic pressure and nearly continuous fluid flow (Carver and Heath, 1998a). The compression/perfusion system was modified to allow

medium to enter the top of the reaction vessel and exit the bottom to prevent the cell/polymer constructs from being entrained in the fluid flow and moving to the top of the vessel. Three tissue baskets were transferred to each reaction vessel and the vessels were prepared as previously described (Carver and Heath 1998b). Medium was continuously perfused through the vessels (10 ml total volume each) at a rate of approximately 3 ml/min during all times when the reactors were not pressurized (22 hours/day). Except for the controls, each reactor was pressurized intermittently (5 sec on/15 sec off) at 500 psi for 20 minutes every 4 hours (2 hours of pressurization per day).

Experimental Design

Five separate experiments were performed to determine the effects of mixed and pressurized culture environments, combined and alone, on *de novo* chondrogenesis. Tissue constructs were cultured in spinners for one, two, and four weeks and then transferred to the compression/perfusion system for up to a total of six weeks. Constructs were also cultured in spinners for the entire six weeks and in the perfusion system without pressurization (control) for five weeks, after one week in spinner flasks. Details of the five experiments and corresponding nomenclature are listed in Table I.

Table I. Experimental design and designated nomenclature.

Experiment Label	Weeks in Spinner Flasks	Weeks in Perfusion Reactor with Intermittent Pressure
1s5c	1	5 (without pressure)
1s5p	1	5
2s4p	2	4
4s2p	4	2
6s	6	0

Construct Analysis

Tissue constructs were examined at four, five, and six weeks for each of the five experiments. Two compression/perfusion reactors (12 constructs) were harvested each week for qualitative and quantitative analysis. Two constructs were used for each assay and compression testing; the remainder of the constructs were prepared for microscopy. Two tissue baskets were harvested each week from the spinner flasks. Two-thirds of a construct was used for each form of quantitative analysis and the remainder was used for microscopy.

Cells. The number of chondrocytes per construct was determined in quadruplicate from a standard curve by fluorometrically measuring the amount of DNA in papain digests using the dye Hoechst 33258 (Kim et al., 1988). With calf thymus DNA as the standard, cell numbers were calculated using a concentration of 7.7 pg DNA/chondrocyte.

GAG. A modified version of the method of Farndale et al. (1986) was used to measure in quadruplicate the amount of GAG in the tissue constructs. Samples were desiccated, weighed, and digested for 24 hours with papain. One ml of 1,9-dimethyl-methylene blue (Aldrich) was added to 20 μ l of the digestate, mixed, and the absorbance read at 525 nm. Chondroitin sulphate b from bovine mucosa (Sigma) was used as the standard.

Collagen. Total collagen content was determined in triplicate from the hydroxyproline concentration. The samples were hydrolyzed with 6 N HCl for 18 hours at 115°C and then reacted with chloramine-T and *p*-dimethylaminobenzaldehyde as previously described (Woessner, 1961).

Compressive Modulus. The compressive modulus, which is a measure of the stiffness or strength of the material, was determined from stress versus strain data obtained with a

Dynamic Mechanical Analyzer (DMA-7, Perkin-Elmer) equipped with a parallel plate sample holder. The compressive modulus was determined from Pyris software for Windows (Perkin Elmer) as the slope at 1% strain (0-2% tested over a period of not more than five minutes).

Electron Microscopy. Tissue constructs were assessed qualitatively using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Constructs were washed with phosphate buffered saline (PBS, 0.1 M, pH 7; Sigma) and fixed in 2% paraformaldehyde/3% glutaraldehyde for 48 hours. After three washes with PBS, the samples were rinsed and immersed in 1% osmium tetroxide in the same buffer for two hours. After rinsing with PBS and water, the samples were dehydrated through a graded alcohol series up to 100% ethanol. SEM samples were critical point dried with carbon dioxide and sputter coated with gold/palladium. TEM pieces were placed into acetone as a transition fluid then embedded in a resin of succinic anhydride, nadic methyl anhydride, Embed resin and 2,4,6-tri(dimethylaminomethyl)phenyl. The resin was polymerized by placing the samples in a 60°C oven for two days. The resin blocks were cut into 50-80 nm thick sections with a diamond knife and stained with lead citrate and uranyl acetate. The samples were viewed on a JEOL 5800LV for SEM and on a JEOL 1200 EX II scanning transmission electron microscope for TEM.

Statistics. Data are presented as means \pm pooled standard deviations for samples analyzed within a given week. Significant differences were determined using the Student's *t* test; all error was calculated with $p < 0.05$.

Results

Cell Concentrations

At week four, the concentration of cells in all five experiments (Table II) was within or above the native regime, which ranges from 4×10^7 to 6×10^7 cells/g tissue (wet weight) in the stifle joint of juvenile horses (Carver and Heath, 1998b). By week six, the 1s5c and 1s5p constructs retained cell concentrations within or very near the native regime, while the others either dropped below (4s2p) or increased above (2s4p and 6s) the native range. There were no clear trends of cell density variation with culture regimen.

Table II. Cell concentrations (cells/g tissue $\times 10^{-7}$) in tissue constructs.

Week	1s5c	1s5p	2s4p	4s2p	6s
4	7.89 ± 2.84	6.90 ± 2.01	5.52 ± 2.01	3.98 ± 2.01	8.43 ± 2.84
5	2.47 ± 2.02	NA	9.31 ± 2.02	1.74 ± 2.02	7.55 ± 2.86
6	4.87 ± 2.49	7.09 ± 2.88	13.12 ± 2.49	1.82 ± 3.53	18.63 ± 3.53

Sulfated Glycosaminoglycan Concentrations

The concentration of GAG increased from week four to week six in all five experiments (Fig. 1). The GAG concentration was greatest in the 2s4p construct at each

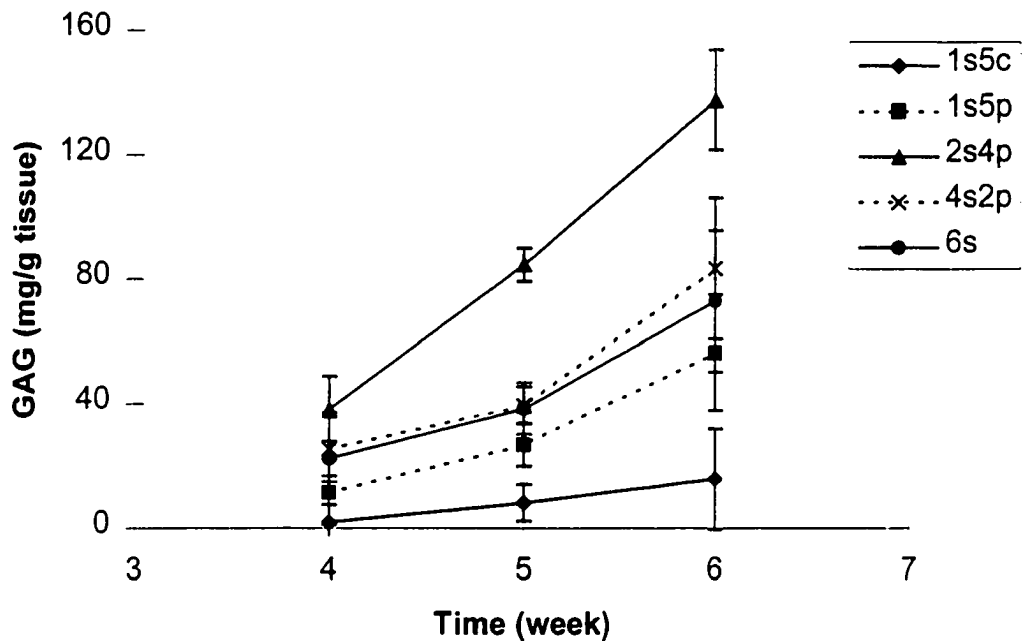


Figure 1. The concentration of GAG increased with culture time in all constructs. Constructs cultured for two weeks or more in spinner flasks contained higher concentrations of GAG, with the combined spinner and compression/perfusion constructs (2s4p) showing the greatest GAG content.

week and, at weeks four and five, significantly exceeded concentrations found in the other experiments by 1.5 to 20 times. At week six, the GAG concentrations in the 1s5p, 4s2p, and 6s constructs were in the native regime for foal articular cartilage (40 to 120 mg GAG/g tissue; Carver and Heath, 1998b) and not significantly different from each other, while concentrations in the 2s4p and 1s5c constructs were slightly above and below native concentrations, respectively.

The average specific production of GAG (mg per 10^7 cells), calculated to account for differences in construct cell density (and the wet and dry weights used as a basis for

calculating cell and GAG concentrations, respectively), confirmed the synergistic effect seen by combining mixing and intermittent pressurization in the 2s4p constructs (Table III). By contrast, no GAG was produced in constructs cultured for four weeks in the spinners, followed by two weeks in the compression/perfusion system (during which the production was measured).

Table III. Average specific production of extracellular matrix between weeks 4 and 6.

Experiment	GAG (mg/10 ⁷ cells)	Collagen (mg/10 ⁷ cells)
1s5c	0.60 \pm 0.60	0.14 \pm 0.12
1s5p	0.96 \pm 0.44	0.56 \pm 0.11
2s4p	2.54 \pm 0.39	0.62 \pm 0.09
4s2p	0.01 \pm 0.90	0.68 \pm 0.35
6s	0.74 \pm 0.37	0.09 \pm 0.05

Total Collagen Concentrations

From week four to week six, the concentration of total collagen increased in all five experiments (Fig. 2). At week six, the collagen concentration was greatest in the 2s4p and 4s2p constructs, and there was no significant difference in concentration between the 1s5p and 6s constructs. The 1s5c constructs contained significantly less collagen at each week than all other constructs, indicating that collagen production was clearly hindered by the

absence of any physical stimulation. All measured collagen concentrations were well below native concentrations (100 to 150 mg/g tissue; Carver and Heath, 1998b).

The small but measurable influence of intermittent pressurization on collagen production can be seen by comparing the average amount of collagen produced per cell between weeks four and six in the five experiments (Table III). Constructs from the three experiments involving intermittent pressurization (1s5p, 2s4p, 4s2p) showed greater specific production of collagen than the two non-pressurized experiments (1s5c, 6s).

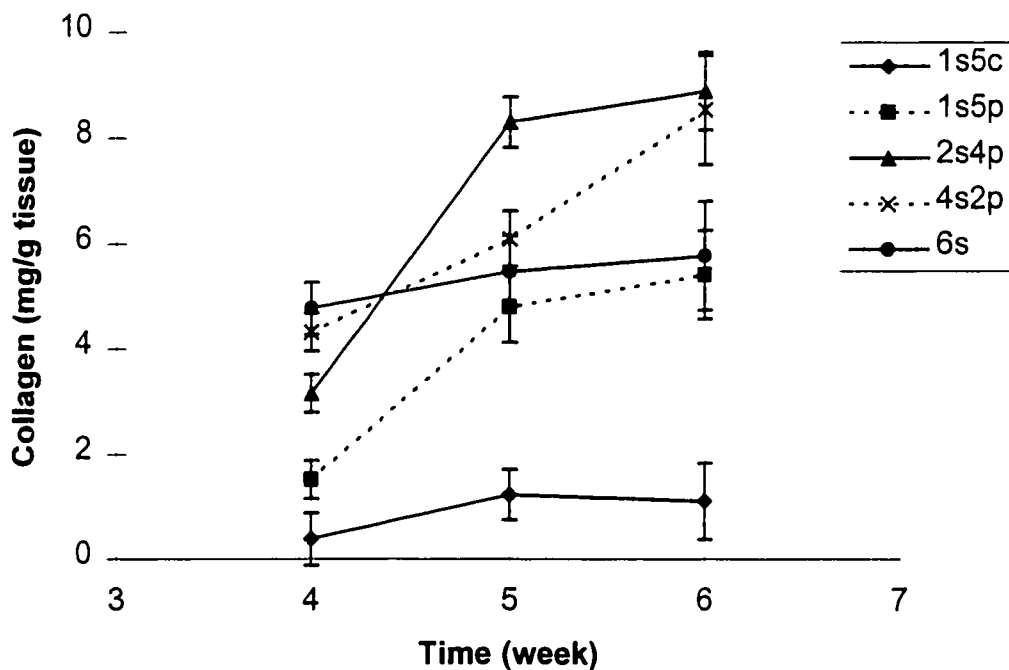


Figure 2. While total collagen concentrations was lowest in the control constructs, the collagen content in all physically stimulated constructs increased with time and were significantly greater than the control. As with GAG, constructs cultured for two weeks or more in spinner flasks contained higher concentrations of collagen, with the combined spinner and compression/perfusion constructs (2s4p and 4s2p) showing the greatest collagen content at six weeks.

Dynamic Mechanical Analysis

While the linear relationships were very similar, the compressive modulus was more strongly correlated with the GAG concentration in constructs which were cultured primarily in a pressurized environment (Fig. 3a) compared to those cultured mainly in a mixed environment (Fig. 3b). None of the constructs reached native levels of the compressive modulus (0.15 to 0.30 MPa; Palmer et al., 1995) despite exhibiting native concentrations of GAG. At week six, the compressive modulus was highest in the 2s4p construct and lowest in the 1s5p construct (Table II). The 1s5c constructs were too fragile to allow determination of the compressive modulus.

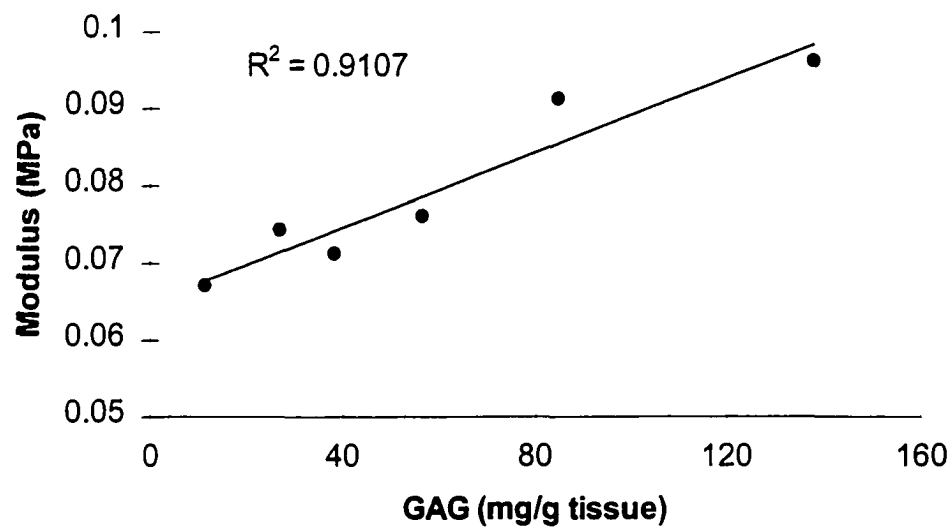


Figure 3a. The compressive modulus versus GAG concentration in pressurized samples.

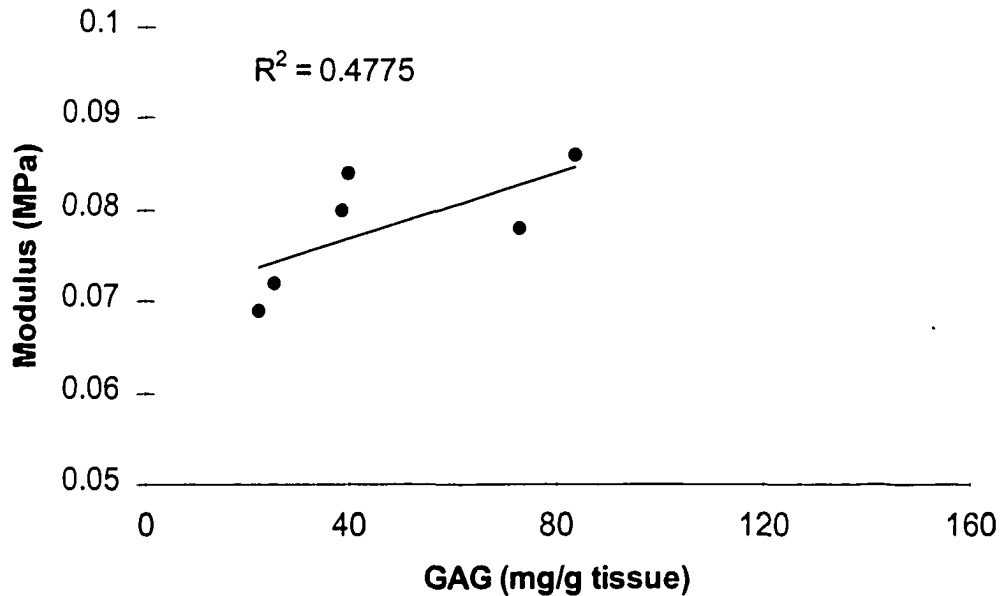


Figure 3b. The compressive modulus versus GAG concentration in mixed samples.

Electron Microscopy

SEM was used to examine cell attachment and ECM formation in tissue constructs after one, two, and four weeks of culture in spinner flasks (Fig. 4). At week one, chondrocytes were adhered to the PGA fibers but little ECM was present (Fig. 4a). At two weeks, the presence of the ECM was clearly evident (Fig 4b) and, at four weeks, the ECM was very dense (Fig. 4c). At the ultrastructural level, TEM showed the orientation of collagen around a chondrocyte (Fig. 5a) and proteoglycan intertwined among collagen fibrils (Fig. 5b) in a six week 2s4p construct.

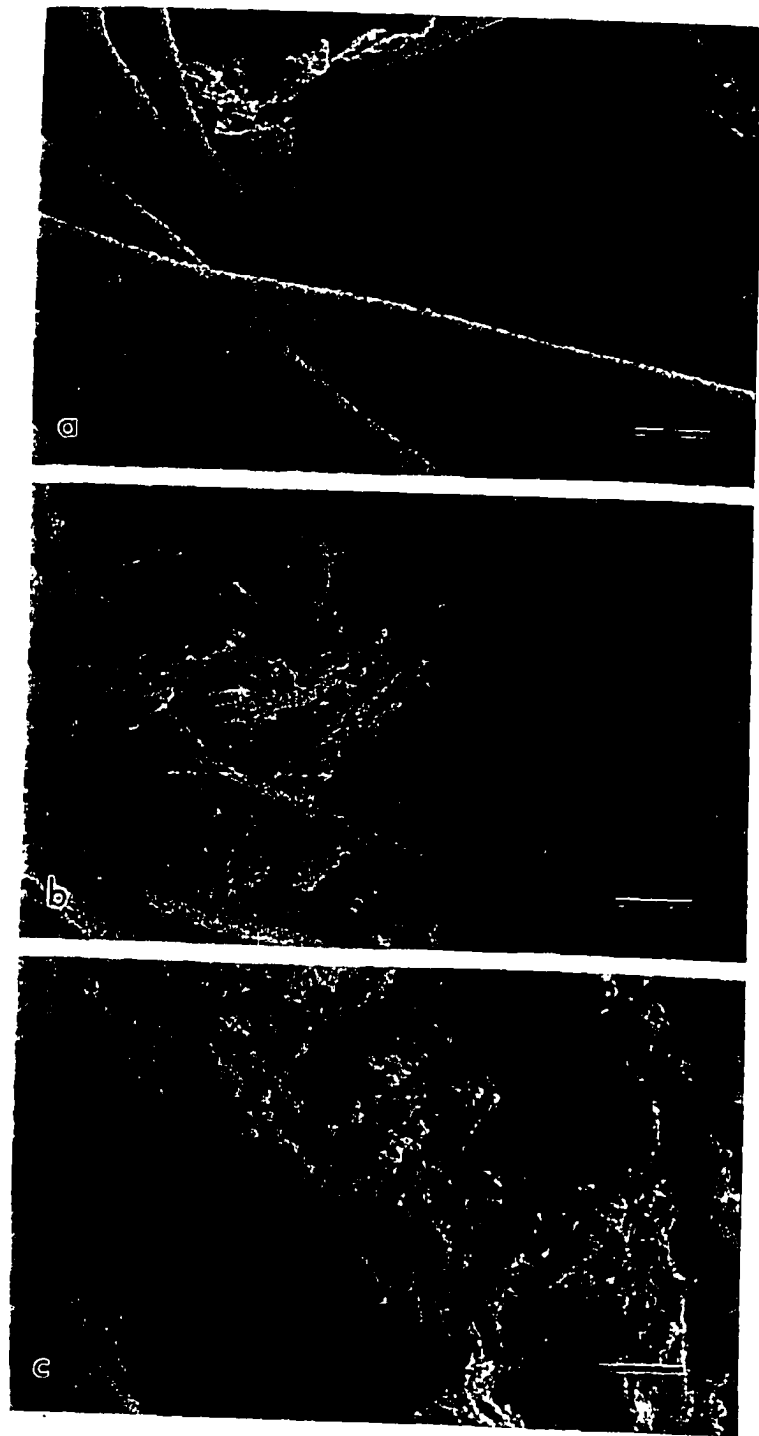


Figure 4. Scanning electron micrographs of 6s constructs harvested at (a) 1 week, (b) 2 weeks, and (c) 4 weeks. All samples were coated with gold/palladium and photographed at 150 X and 10 kV. Each bar represents 30 μm .

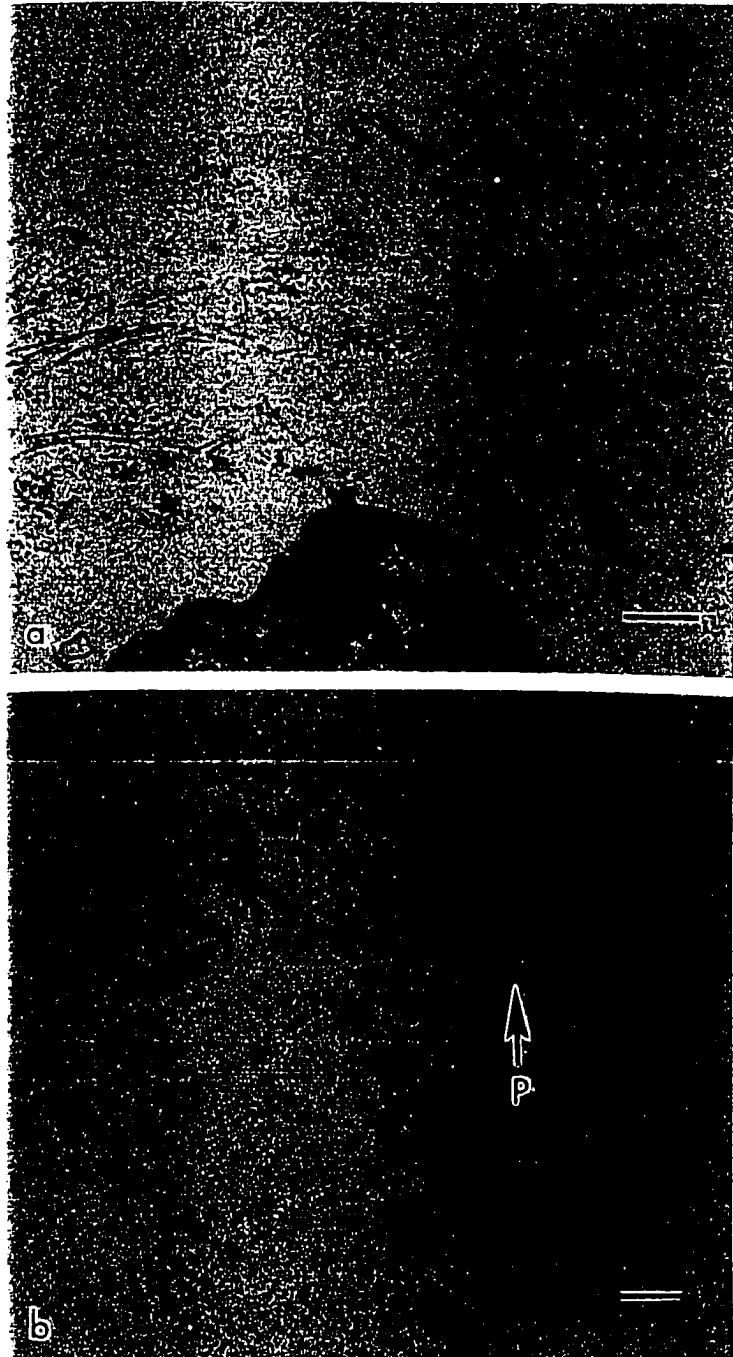


Figure 5. Transmission electron micrographs show (a) the collagen orientation around a chondrocyte at 10,000 X (bar = 0.5 μ m), and (b) proteoglycan (P) at 40,000 X (bar = 0.1 μ m) in a 2s4p construct. All sections were stained with lead citrate and uranyl acetate.

Discussion

In the past decade there have been many advances in understanding the conditions necessary to develop tissue constructs for replacement of damaged or diseased articular cartilage. To be useful *in vivo*, these grafts must possess the same biochemical composition and biomechanical properties as healthy native tissue. Our work to date has focused on determining the effects of physical stresses (compression and shear) on development of cartilaginous tissue constructs from isolated chondrocytes embedded in a cell/polymer matrix.

Dynamic compressive loading has been shown many times to increase the production of GAG, a component of the proteoglycan macromolecule, by articular chondrocytes in cell cultures and explants over the short term (Parkkinen et al., 1993; Buschmann et al., 1995). Using a reaction system which incorporates many of the same conditions as an articulating joint (physiological levels of loading, long-term culture, and a suitable 3-D growth environment), this work and others (Carver and Heath, 1998a,b) have demonstrated that dynamic compression also increases GAG production in tissue constructs during long term culture. Well-mixed systems also support development of tissue constructs with GAG concentrations in the native regime (Freed and Vunjak-Novakovic, 1997) indicating that either compression or shear, the primary forces experienced in the native joint, can be used to stimulate GAG production by chondrocytes. An additional advantage of well-mixed systems (Freed and Vunjak-Novakovic, 1997) is that they support development of tissue constructs which are larger in size and mass than those cultured only in pressurized systems (Carver and

Heath, 1998b). As demonstrated in this study, tissue constructs with higher concentrations of GAG and collagen, greater mass, and mechanical properties more like those *in vivo* than those cultured with hydrostatic compression or fluid shear alone can be produced by combining these forces, suggesting a synergistic relationship which may contribute to the development of cartilaginous tissue. Simultaneous shear and compression, as experienced *in vivo*, may provide the optimum environment.

Although the tissue constructs developed in this study had the same concentration of GAG as native tissue, the values of the compressive modulus were below the native range. Earlier studies have shown that high proteoglycan content is correlated with high compressive modulus (Kempson et al., 1980; Akizuki et al., 1986). This apparent inconsistency suggests that concentration alone may not be a good indicator of tissue mechanical strength in immature tissues or tissue constructs. The molecular structure of GAG (and collagen) is also an important component of the tissue's ability to withstand repeated dynamic loading (Mow et al., 1984). The low values (by a factor of two or more) of the compressive modulus compared to native values observed in this study suggest either that the GAG had not aggregated to the extent found *in vivo* and/or that the low levels of collagen, which serves to constrain the aggregated proteoglycan molecules, limited the ability of the tissue to resist compression. Whether or not physical forces affect GAG aggregation and/or association with collagen is still under investigation; however, we have repeatedly seen a strong correlation between the compressive modulus and the concentration of GAG only in pressurized samples (Carver and Heath, 1998a) suggesting that intermittent pressure at physiological levels may influence structure as well as content during *de novo*

chondrogenesis. Addressing concerns such as these is critical to the development of functional grafts.

Production of native levels of collagen in tissue constructs has continued to be an obstacle despite recent advances. The production of collagen by articular chondrocytes increases when cultured on collagen (Grande et al., 1997), with growth factors such as TGF- β (Zimmer et al., 1995), by increasing the cell seeding density (Vunjak-Novakovic et al., 1998), with increasing levels of intermittent pressurization (Carver and Heath, 1998b), and, as shown in this work, in a combination of well-mixed and pressurized growth environments. Using the compression/perfusion system (Carver and Heath, 1998a), it is possible to culture tissue constructs with a combination of these conditions for long time periods so that collagen production by articular chondrocytes can be maximized and constructs are allowed adequate time to fully develop. Whether this will be sufficient to regenerate constructs with native concentrations of ECM components and biomechanical properties remains to be seen.

Combining mixing and intermittent pressure results in higher concentrations of GAG and collagen in tissue constructs than application of either condition alone, suggesting that the optimum culture environment for *de novo* chondrogenesis should include both. While mixing has already been noted for its ability to promote seeding and distribution of cells throughout the polymer matrix (Vunjak-Novakovic et al., 1996), culture of cell/polymer constructs in spinner flasks beyond the time necessary for seeding may provide additional benefits which result from convective mass transfer. SEM micrographs of constructs from spinner flasks show cells adhered to the PGA but little ECM at one week; by two weeks, the presence of the ECM is obvious. By four weeks, the ECM appeared much denser than at two

weeks; quantitatively, the GAG and collagen concentrations in the 6s constructs at four weeks were at or near the highest of the five experimental conditions. By six weeks, however, the constructs cultured in a combination of environments had higher concentrations of GAG (2s4p) and collagen (2s4p, 4s2p) than those grown in spinners alone (6s). Culture of constructs for up to two weeks in spinner flasks prior to transferring them to the compression/perfusion system enhanced *de novo* chondrogenesis. Perhaps the well-mixed environment of the spinner flask facilitated early development of the ECM, the presence of which enhanced the response of the construct to intermittent compression. In a similar fashion, dynamic compression of chondrocytes immobilized in an agarose gel resulted in increased rates of matrix formation at higher concentrations of the ECM (Buschmann et al., 1995).

Conclusions

Development of cartilaginous tissue constructs in a combination of mixed and pressurized environments resulted in greater concentrations of GAG and collagen than culture with mixing or intermittent pressure alone. The highest concentrations and productivities of GAG and collagen were found almost exclusively in constructs cultured for two or four weeks in spinner flasks followed by intermittent pressurization with perfusion. Well-mixed culture promoted cell attachment and distribution of cells within the polymer matrix as well as development of the ECM, which was confirmed by SEM. Subsequent culture with intermittent pressure appeared to accelerate the production of GAG and collagen suggesting that the presence of a partially developed ECM enhanced the cellular response to

compression. Finally, the compressive modulus was directly proportional to GAG concentration in all physically stressed constructs but with a stronger correlation for constructs developed primarily in the pressurized environment. Physical forces may influence not only the composition but also the structure of developing tissue and may be critical to the production of functional biological grafts.

Acknowledgments

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CHAPTER 5. GENERAL CONCLUSIONS

General Discussion

The long-term goal of this project was to develop regenerated articular cartilage *in vitro* most like that found *in vivo* in the shortest time possible. Of particular interest was the effect of physiological intermittent pressurization on the production of the extracellular matrix (ECM) by articular chondrocytes since articular cartilage *in vivo* is frequently loaded in a dynamic manner. In order to fulfill this goal, a cell culture system was designed and constructed which was capable of delivering physiological levels of hydrostatic pressurization in an intermittent manner to regenerating cell/polymer constructs. The challenge in creating a reaction system which could effectively create native-like tissue was combining many different culture conditions which had previously been determined individually, such as a three-dimensional growth environment, long-term cultures, and dynamic loading similar to that found *in vivo*. Nonwoven meshes of polyglycolic acid (PGA) have been quite effective in providing chondrocytes with the three-dimensional environment they need to produce extracellular matrix components normally found *in vivo* [1-6]. This particular polymer was used in our experiments because it is biodegradable, resorbable, easily formed into given shapes, and commercially available. Intermittent pressurization [7] and physiological hydrostatic pressurization [8] have both been found to increase the production of the extracellular matrix. Because of the nutrient requirements by the cells, however, studies which were able to previously combine these conditions were limited to short culture times (up to 48 hours) which is not long enough to fully develop a regenerated

construct [9]. In order to increase the culture time, medium must be replaced on a regular basis [10]. By combining these key culture conditions we were able to effectively develop *de novo* tissue with many of the characteristics of native tissue.

Because a reaction system like ours had never been developed before, many experiments were performed to optimize the culture conditions. Glucose and oxygen uptake experiments were performed to model the nutrient requirements by isolated articular chondrocytes, allowing us to determine how often fresh medium must be perfused through the reaction space. In our initial experiments, medium was only perfused through each reactor for a total of 18 minutes every day to fulfill the minimum nutrient requirements of the isolated cells; the reactors remained stagnant the remainder of the time. Excess medium perfusion was unnecessary to show the effects of physiological intermittent pressurization on the production of the ECM. Initial experiments were performed to show that the system could be used to culture regenerating tissue and to what extent the application of pressure would enhance the production of the ECM.

Adult chondrocytes were used in the first few experiments because the ability to regenerate native articular cartilage with these cells would be advantageous. From a practical standpoint, regenerating articular cartilage to be used as a replacement tissue would be quicker and safer if subjects needing the tissue could donate their own cells because the tissue would be readily available and the chance of rejection would be reduced. Typically, the cells found within osteoarthritic cartilage are no longer capable of regenerating the extracellular matrix. As an animal grows, its bones elongate, with the development of articular cartilage preceding bone formation. When a bone reaches its maximum length the

epiphyseal plate closes, and the chondrocytes making up the cartilage may exhibit a decrease in their metabolism. Since cartilage production is no longer necessary when a joint is fully developed, the chondrocytes within the cartilage may start focusing on maintaining the tissue. If there was some way to increase the rate of matrix production above that required for normal maintenance then regenerating native tissue would become possible. Whether intermittently applied physiological pressures might accomplish this with adult cells was unknown. Our results indicated that, although intermittent pressurization did seem to stimulate adult cells to produce the ECM, the concentrations of the ECM were much greater in constructs created using foal cells. Since native concentrations of the ECM were never obtained using adult cells, foal cells will most likely be used for all future experiments.

Physiological intermittent pressurization was found to increase the concentration of sulfated glycosaminoglycan (s-GAG) in regenerated constructs at both pressures tested (500 and 1000 psi), and increasing the level of pressure was found to further increase the concentration of s-GAG. Using dynamic loading to increase s-GAG production by articular chondrocytes over short time periods has been described by many researchers [11-14]. Since we noted the same trends in our system over long time intervals, we felt confident that our system was working as predicted. For the first time we saw an increase in the concentration of collagen in constructs cultured at 1000 psi. Since this increase was not noted in 500 psi pressurized or control constructs in stagnant cultures, this indicated that some minimum level of dynamic force may be needed to stimulate collagen production by articular chondrocytes.

Although stagnant (minimum required medium perfusion) cultures were effective in demonstrating the effects of pressure on matrix production, they typically produced

regenerated tissue which was smaller in size and mass than regenerated constructs cultured in mixed medium systems due to the diffusional limitations of the cellular metabolites. By modifying our system setup, we were able to combine mixing with pressurization, something which has never been done before with regenerating cartilage cultures. When samples were isolated after being cultured in a mixed and pressurized environment, the benefits of mixing were noticed immediately. The average construct mass and size was two to three times larger than those harvested in stagnant pressurized cultures and, since we could culture twice as many constructs, the total mass isolated from each reactor was seven to eight times larger. When comparing the concentrations of the extracellular matrix in stagnant and mixed pressurized cultures, the benefits of mixing might not be immediately noticeable (see Fig. 1 and Fig. 2). Both of these data sets were analyzed at 4, 5, and 6 weeks total culture time for constructs

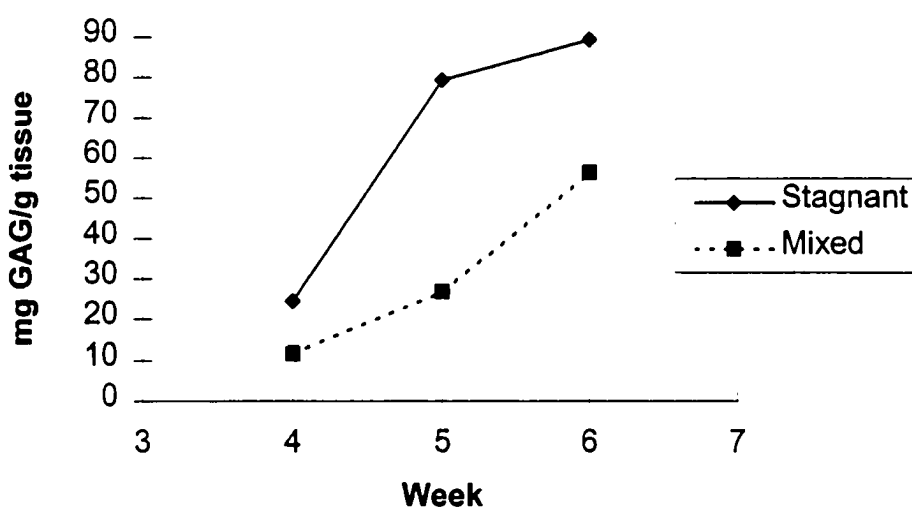


Figure 1. GAG concentrations in stagnant and mixed pressurized cultures up to 6 weeks.

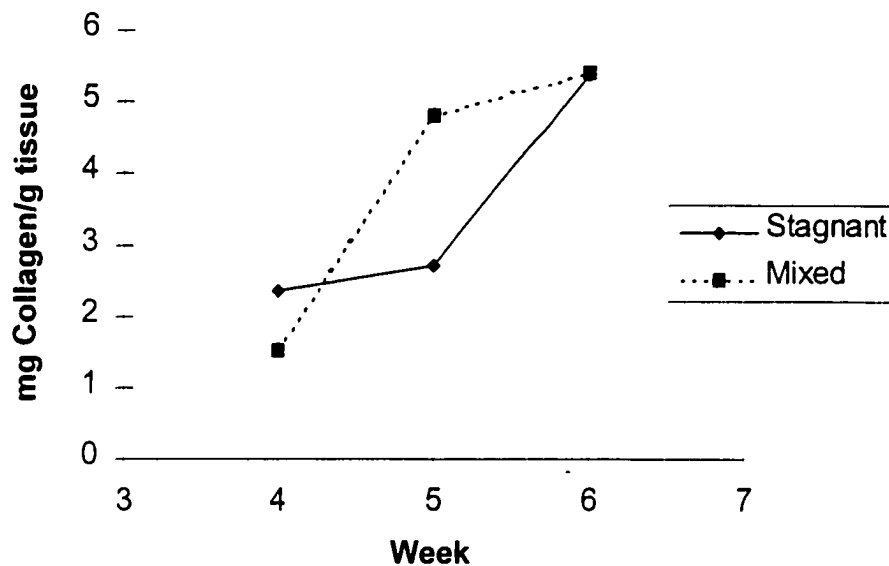


Figure 2. Collagen concentrations in stagnant and mixed pressurized cultures up to 6 weeks.

dynamically seeded for approximately one week and pressurized at 500 psi up to 6 weeks (stagnant cultures previously reported ECM concentrations after 3, 4 and 5 weeks of pressurized culture, not including the week of dynamic seeding). Although the concentration of GAG was greater in stagnant cultures after 6 weeks, both constructs had native concentrations of GAG and, once again, the mixed samples were two to three times as large as stagnant samples. There appears to be no effect of mixing on the concentration of collagen after 6 weeks even though the total collagen content per sample was greater in mixed samples. One reason the concentrations of the extracellular matrix components in regenerated samples may have appeared different between stagnant and mixed pressurized cultures was that the fluid velocity through the reaction space was much lower than that

experienced in spinner flasks. In order for regenerating samples to experience an equivalent flow in each reactor equivalent to that of constructs isolated within spinner flasks, a flow rate of approximately 1,600 ml/min must be used. At this time, flow rates that large are not possible in our reactors. The maximum flow rate we can use, which allows us to maintain a stable pressurized environment, is only approximately 3 ml/min. Even though this value is two orders of magnitude lower than that in spinner flasks, it does effectively provide the isolated cells with the nutrients they need for normal cellular metabolism. Apparently, if the constructs are cultured in a mixed environment (spinner flasks) for long enough to establish an initial matrix, they do not require large flow rates once transferred to the pressurized environment.

When pressurized and mixed culture environments were combined, the concentration of collagen, even at 500 psi, was greater than in constructs cultured in a single environment. This was not seen in stagnant cultures at 500 psi but was observed in stagnant cultures at 1000 psi. We had previously concluded that some minimum level of dynamic force may be needed to stimulate collagen production, a conclusion which still holds true. By combining compressive and shear forces, collagen production can be stimulated in the same manner as increasing the level of compressive force. Most likely increasing the compressive force above 500 psi in cultures combining pressure and mixing will further increase the concentration in collagen found in regenerated constructs. However, cell/polymer constructs may require additional time in a mixed environment so that the initial matrix that develops can withstand an increase in compressive force.

Since a correlation between the compressive modulus and the concentration of sulfated glycosaminoglycan was only observed in samples cultured mostly in a pressurized environment, the application of pressure to regenerating constructs may influence the structural development of the tissue in ways that different types, or the absence, of force cannot. Other studies involving mechanical compression have reported simultaneous increases in GAG content and modulus levels, however connections between the two were not discussed [15]. One possible explanation is that intermittent pressurization influences aggregation of GAG, which would help form a stronger, more cohesive matrix. Whether or not the GAG produced in regenerated samples has aggregated to the same extent as tissue found *in vivo* is unlikely, and, if it has not, could be one of the reasons that regenerated samples have yet to reach compressive strengths normally exhibited by native tissue. Until a regenerated tissue can be developed that can repeatedly withstand physiological loading, graft tissue created *in vitro* and implanted *in vivo* will not be functional. From our studies, it appears that pressurization is needed to produce mechanically stable tissue.

Recommendations for Future Research

One of the advantages of the reaction system developed for our studies is that it can be set up in a number of different operating configurations. This allows the user to explore the effects of many different culture conditions on regenerating articular cartilage *in vitro*. For example, changes in frequency of pressure, level of pressure, medium flow rate, type of medium, and type of biomaterial carrier can be easily accomplished. From our studies up to now, many key culture conditions have been investigated which produce regenerated

constructs most like that found *in vivo* and should not be changed. Foal articular chondrocytes should be used in order to maximize the production of the extracellular matrix. Dynamic mixing should also be used to establish a stable initial matrix in cell/polymer constructs, however, the time needed to develop a stable matrix should be explored. The modified tissue baskets which were used as support cages in the final experiment (chapter 4) eliminated the need for adhesives within each reactor and, by allowing flow through the basket, created regenerated tissue larger than constructs cultured on steel support cages. These baskets should be used in order to maximize the flow of medium over the regenerating cell/polymer constructs.

When we initially designed the reaction system, there was no need for high flow rates of medium throughout each reactor so a low-flow, multichannel pump was acquired to provide medium to each reactor at the same rate. To achieve greater volumetric flow rates in the reaction space, a new pump must be purchased and each reactor must be modified slightly (larger bore sizes for the “medium in” and “medium out” ports) to allow a more uniform medium flow over the constructs. By increasing the flow through each reactor, regenerated constructs should be larger in size and perhaps have greater concentrations of the extracellular matrix. One detail that needs to be considered before making any changes in the internal volume of each reactor is that increasing the volume will increase the deflection distance of the reaction piston when a reactor is pressurized.

Greater concentrations of the extracellular matrix can be achieved by increasing the cell seeding densities in mixed cultures [16]. In our studies, polymer constructs were seeded between 4 to 7×10^6 cells per construct. At these concentrations, we were able to achieve

native concentrations of GAG and cells. Perhaps increasing the cell seeding densities by a factor of two or more would establish a stable matrix in regenerating constructs cultured in mixed systems in a shorter period of time. Since the cells are the sole producers of the extracellular matrix in regenerating constructs, increasing their concentrations throughout the polymer constructs should decrease the culture time needed to achieve *in vivo* levels of the extracellular matrix.

There are a few forms of analysis which can be used on regenerated constructs which were not used in our investigations. Chromatographic techniques may possibly be used to determine to what extent the GAG produced in tissue developed *in vitro* has aggregated. By also analyzing native and control tissue, the effects of pressurization on the formation of GAG aggregates could be described. Since the assay used to determine the concentration of collagen in regenerated samples only quantified total collagen, the amount of type II collagen (the type comprising native tissue) produced was unknown. Qualitative assessments of collagen type can be made using fluorescence microscopy [6] while quantitative assessments can be made by combining the hydroxyproline assay with cyanogen bromide ratio analysis [17]. One other form of qualitative analysis that can be used to assess regenerated constructs is confocal microscopy. This form of microscopy nondestructively takes optical sections of a sample and allows the user to three-dimensionally reconstruct the sample with computer software. This could be especially useful in determining the distribution of the extracellular matrix throughout regenerated samples.

Perhaps the greatest challenge in regenerating articular cartilage is developing tissue with native concentrations of collagen. As described in chapter 4, there are many separate

influences that can be combined in our system to maximize collagen production. Growth factors, biomaterial carrier type, and some minimal level of dynamic force have all been shown to increase collagen production. It may just be a matter of culturing tissue for a long enough period of time to allow the extracellular matrix to fully develop. Since type II collagen is the main organic component comprising native tissue, it is my opinion that functional regenerated cartilage must consist of *in vivo* collagen concentrations.

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APPENDIX A. RAW DATA

Chapter 2

Table 1. Cell densities found in figure 3.

Week	Control Concentration, $\times 10^7/\text{g}$ tissue	Pressurized Concentration, $\times 10^7/\text{g}$ tissue
3	2.12 ± 0.54	2.51 ± 0.77
4	4.20 ± 0.36	4.71 ± 0.26
5	7.29 ± 2.51	5.18 ± 2.51

Table 2. GAG concentrations found in figure 4.

Week	Control Concentration, mg/g tissue	Pressurized Concentration, mg/g tissue
3	4.40 ± 1.76	24.45 ± 1.76
4	40.19 ± 17.78	79.35 ± 12.57
5	19.57 ± 42.45	89.28 ± 42.45

Table 3. Compressive modulus' and GAG concentrations found in figure 5.

Data Point	Compressive Modulus, MPa	GAG Concentration, mg/g tissue
1	0.012	24.45
2	0.063	79.35
3	0.057	89.28

Table 4. Collagen concentrations found in figure 6.

Week	Control Concentration, mg/g tissue	Pressurized Concentration, mg/g tissue
3	3.38 ± 0.55	2.34 ± 0.78
4	2.07 ± 0.44	2.71 ± 0.31
5	5.8 ± 2.3	5.4 ± 2.3

Chapter 3

Table 1. Foal GAG concentrations found in figure 1a.

Week	1000 psi Sample Concentration, mg/g tissue	500 psi Sample Concentration, mg/g tissue	Control Sample Concentration, mg/g tissue
3	99.49 ± 2.22	24.45 ± 1.81	3.21 ± 1.34
4	115.30 ± 19.02	79.35 ± 15.53	29.53 ± 14.37
5	133.70 ± 38.51	89.28 ± 31.44	25.95 ± 24.35

Table 2. Adult GAG concentrations found in figure 1b.

Week	1000 psi Sample Concentration, mg/g tissue	500 psi Sample Concentration, mg/g tissue	Control Sample Concentration, mg/g tissue
3	1.17 \pm 2.56	8.21 \pm 1.81	2.37 \pm 1.48
4	1.44 \pm 2.02	5.23 \pm 1.42	1.76 \pm 1.42
5	3.47 \pm 1.42	5.72 \pm 1.01	2.04 \pm 1.01

Table 3. Foal collagen concentrations found in figure 2a.

Week	1000 psi Sample Concentration, mg/g tissue	500 psi Sample Concentration, mg/g tissue	Control Sample Concentration, mg/g tissue
3	5.93 \pm 1.03	2.48 \pm 1.03	2.98 \pm 0.59
4	9.88 \pm 0.64	3.04 \pm 0.45	2.01 \pm 0.45
5	11.93 \pm 2.70	6.66 \pm 1.91	6.30 \pm 1.56

Table 4. Adult collagen concentrations found in figure 2b.

Week	1000 psi Sample Concentration, mg/g tissue	500 psi Sample Concentration, mg/g tissue	Control Sample Concentration, mg/g tissue
3	1.69 \pm 0.35	1.14 \pm 0.50	1.67 \pm 0.29
4	4.91 \pm 1.20	1.25 \pm 0.85	1.24 \pm 0.85
5	7.31 \pm 0.45	3.04 \pm 0.32	0.45 \pm 0.32

Chapter 4

Table 1. GAG concentrations found in figure 1.

Week	"1s5c" Concentration, mg/g tissue	"1s5p" Concentration, mg/g tissue	"2s4p" Concentration, mg/g tissue	"4s2p" Concentration, mg/g tissue	"6s" Concentration, mg/g tissue
4	1.88 ± 14.77	11.47 ± 10.45	38.26 ± 10.45	25.33 ± 10.45	22.28 ± 14.77
5	8.13 ± 5.93	26.76 ± 6.85	84.60 ± 5.93	39.46 ± 5.93	38.41 ± 8.39
6	15.76 ± 16.08	56.33 ± 18.59	137.69 ± 16.08	83.48 ± 22.74	72.87 ± 22.74

Table 2. Collagen concentrations found in figure 2.

Week	"1s5c" Concentration, mg/g tissue	"1s5p" Concentration, mg/g tissue	"2s4p" Concentration, mg/g tissue	"4s2p" Concentration, mg/g tissue	"6s" Concentration, mg/g tissue
4	0.38 ± 0.50	1.51 ± 0.36	3.15 ± 0.36	4.31 ± 0.36	4.77 ± 0.50
5	1.23 ± 0.48	4.80 ± 0.68	8.30 ± 0.48	6.09 ± 0.53	5.47 ± 0.68
6	1.10 ± 0.73	5.41 ± 0.84	8.88 ± 0.73	8.53 ± 1.03	5.77 ± 1.03

Table 3. Compressive modulus' and GAG concentrations found in figure 3a.

Data Point	GAG Concentration, mg/g tissue	Compressive Modulus, MPa
1	11.47	0.067
2	26.76	0.074
3	38.26	0.071
4	56.33	0.076
5	84.60	0.091
6	137.69	0.096

Table 4. Compressive modulus' and GAG concentrations found in figure 3b.

Data Point	GAG Concentration, mg/g tissue	Compressive Modulus, MPa
1	22.28	0.069
2	25.33	0.072
3	38.41	0.080
4	39.46	0.084
5	72.87	0.078
6	83.48	0.086

Table 5. Dry weights of 6 week samples in all five experiments. IPPR values are from chapter 3 experiments.

Culture Environment	Dry Weight, mg
IPPR, Foal at 500 psi	3.6
IPPR, Foal at 1000 psi	3.0
1 spin/5 control	4.1
1 spin/5 pressure	4.6
2 spin/4 pressure	6.2
4 spin/2 pressure	6.8
6 spin	8.4

APPENDIX B. DIFFUSIONAL ANALYSIS

The following is an order of magnitude analysis to compare the volumetric fluxes through the cell/polymer scaffolds in the three separate culture environments used for this work (compression/perfusion reactors with no flow, compression/perfusion reactors with nearly continuous fluid flow, spinner flasks with turbulent flow).

Compression/perfusion reactors with no flow – pure diffusion

For pure diffusion, estimate the mass flux as:

$$J_m = D_{\text{eff}}(\Delta C/\Delta L) \quad (1)$$

The effective diffusivity can be defined as:

$$D_{\text{eff}} = D_{\text{bulk}}(\epsilon/\tau) \quad (2)$$

where ϵ is the polymer porosity and τ is the tortuosity. Using oxygen as the limiting nutrient (solute) and water as the solvent, the bulk diffusivity at 37 °C is $4.76 \times 10^{-5} \text{ cm}^2/\text{sec}$.

Assuming the tortuosity is approximately 1 for a porosity of 0.97, equation (2) becomes:

$$D_{\text{eff}} = 4.76 \times 10^{-5} \text{ cm}^2/\text{sec}(0.97/1) = 4.62 \times 10^{-5} \text{ cm}^2/\text{sec}$$

Each polymer pad is 0.5 cm thick with medium on all sides so that the maximum distance oxygen has to diffuse is $\Delta L = 0.25 \text{ cm}$. Assuming that the concentration of oxygen in the middle of each pad is approximately 0 and the concentration of oxygen at the surface is the solubility of oxygen in water ($8 \times 10^{-6} \text{ g/cm}^3$), equation (1) becomes:

$$J_m = 4.62 \times 10^{-5} \text{ cm}^2/\text{sec} (8 \times 10^{-6} \text{ g/cm}^3/0.25 \text{ cm})$$

$$J_m = 1.48 \times 10^{-9} \text{ g/(cm}^2 \cdot \text{sec)}$$

Dividing the mass flux by the density of gaseous oxygen gives the volumetric flux as:

$$J_v = [1.48 \times 10^{-9} \text{ g/(cm}^2 \cdot \text{sec)}] / 1.43 \times 10^{-3} \text{ g/cm}^3$$

$$J_v = 1.04 \times 10^{-6} \text{ cm/sec}$$

Compression/perfusion reactors with nearly continuous fluid flow – Darcy's Law

Using Darcy's Law for estimating the volumetric flux of a solute through a polymer membrane, the volumetric flux is the same as the velocity of the solute through the pad. The maximum velocity through the reactors can be calculate by dividing the volumetric flow rate

by the cross-sectional area of each reactor. Assuming a flow rate of 5 ml/min and a cross-sectional area of $0.5\pi \text{ cm}^2$, the velocity (and volumetric flux) is:

$$v \text{ (or } J_v) = (5 \text{ cm}^3/\text{min}) / (60 \text{ sec/min} * 1.57 \text{ cm}^2) = 5.31 \times 10^{-2} \text{ cm/sec}$$

Spinner flasks with turbulent flow – Darcy's Law

Use Darcy's law once again to estimate the volumetric flux as the fluid velocity through the pad. Assume the fluid velocity is the same as the speed of the impeller edge which mixes the medium. The distance the this edge cover is:

$$d = 2\pi r = 2 * \pi * 2 \text{ cm} = 12.57 \text{ cm}$$

At 50 rpm's the velocity is estimated to be:

$$v \text{ (or } J_v) = 12.57 \text{ cm} (50 \text{ rpm}/60 \text{ sec}) = 10.5 \text{ cm/sec}$$

Conclusions

The flux in the reactors with fluid flow is approximately 4 orders of magnitude of pure diffusion alone. The flux in the spinners is approximately 3 orders of magnitude above the reactor with fluid flow and 7 orders of magnitude above pure diffusion. All fluxes calculated were the highest values possible and could likely be up to an order of magnitude lower. All fluxes will decrease as the polymer fills with extracellular matrix.

ACKNOWLEDGMENTS

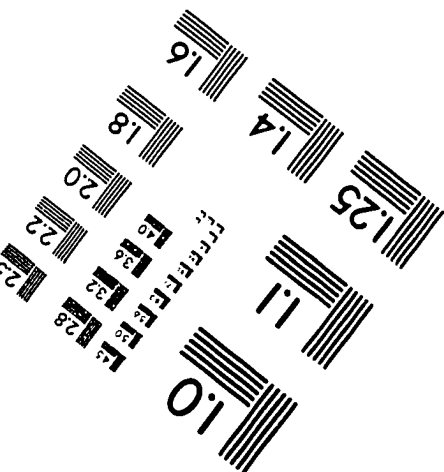
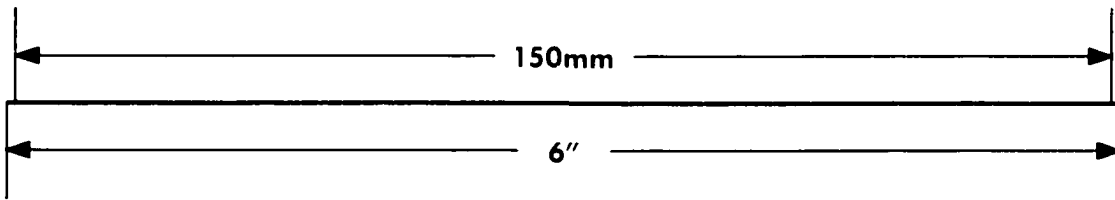
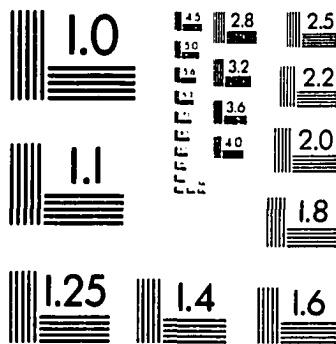
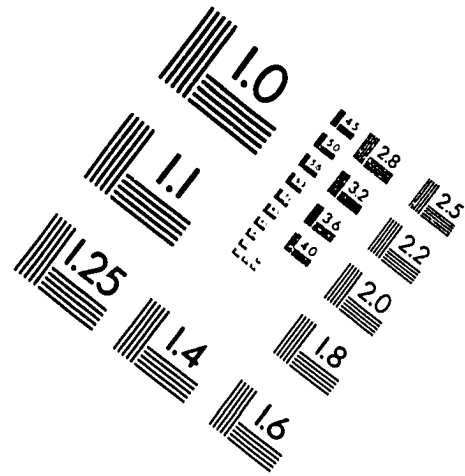
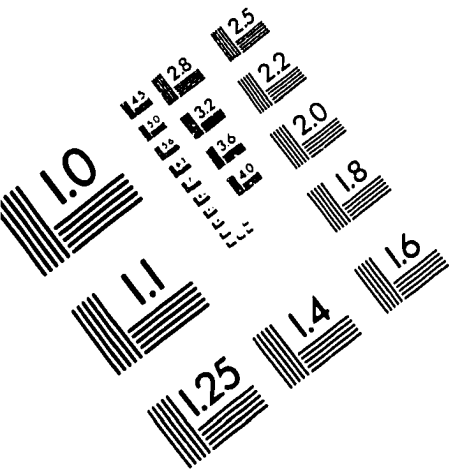
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IMAGE EVALUATION TEST TARGET (QA-3)



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